UNIVERSITY OF ALBERTA

EFFECTS OF INTRA-CEREBRAL INFUSION OF ANXIOLYTIC COMPOUNDS IN ANIMAL MODELS OF ANXIETY

BY

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ABSTRACT

Previous work has shown that eliminating or suppressing activity in the septal nucleus reduces rats' fear responses in two animal models of anxiety; i.e., septal lesions or intra-septal infusions of the benzodiazepine receptor agonist, midazolam, suppress rats' open-arm avoidance in the elevated plus-maze and their burying behavior in the shock-probe burying test (Chapter 1). Despite this apparent correspondence between the neural mechanisms of open-arm avoidance and burying behavior, the current results suggest that these specific fear responses may be differentially governed by functionally distinct subsystems within the septal nucleus. In the first series of experiments (Chapter 2) septal infusions of the 'inhibitory' 5-HT_{1A} receptor agonist, R(+)-8-OH-DPAT profoundly suppressed rats' burying behavior in the shock-probe burying test, without altering their normal open-arm avoidance in the elevated plus-maze. Septal regulation of these different fear reactions was similarly dissociated following local application of excitatory amino acid receptor antagonists into the septal nucleus (Chapter 3). Specifically, the NMDA receptor antagonist, AP-5, dramatically reduced burying behavior but failed to alter open-arm avoidance, whereas septal infusions of the non-NMDA receptor antagonist, CNQX, reduced fear responding in both tests. The neural control of open-arm avoidance and burying behavior was also dissociated following local inhibition of a primary input structure to the septal nucleus; i.e., dorsal hippocampal infusions of R(+)-8-OH-DPAT or midazolam selectively increased open-arm exploration without altering burying behavior (Chapters 2 and 4). Finally, septal infusion of L-glutamate selectively suppressed the open-arm exploration of rats previously infused with midazolam in the dorsal hippocampus, suggesting that the anxiolytic effects of inhibiting dorsal hippocampal

activity may be due to suppressed release of glutamate in the septal nucleus (Chapter 4). Thus, overall, it appears that 5-HT_{1A}, NMDA, and non-NMDA receptors in the septal nucleus might have specific roles in the regulation of different fear responses. Similarly, it appears that specific projection paths coming into the septal nucleus from the dorsal hippocampus differentially regulate rats' fear responses in the plus-maze and burying tests.

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ABBREVIATIONS

AMPA	(±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANCOVA	analysis of covariance
ANOVA	analysis of variance
α	alpha
AP-5	D(-)-2-Amino-5-phosphonopentanoic acid
AP-7	D(-)-2-Amino-7-phosphonoheptanoic acid
BNST	Bed Nucleus of the Stria Terminalis
BZ	benzodiazepine
cm	centimeter
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CPP	(±)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid
5,7-DHT	5,7-dihydroxytryptamine
DPAG	dorsal periaqueductal gray
EAA	excitatory amino acid
EPSP	excitatory postsynaptic potential
g	gram
GABA	γ-aminobutyric acid
L-Glutamate	S(+)-1-Aminopropane-1,3-dicarboxylic acid
h	hour

5-HT	5-hydroxytryptamine (serotonin)
ip	intraperitoneal
kg	kilogram
LSc	Lateral Septum caudal
LSr	Lateral Septum rostral
µg	microgram
µl	microliter
min	minutes
mm	millimeter
mA	milliampere
NMDA	N-Methyl-D-aspartic acid
R(+)-8-OH-DPAT	R(+)-2-Dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene
s.e.m.	standard error of the mean
V	volt

Chapter 1

Introduction

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Introduction

Jeffrey Gray (1982) noted a striking similarity between the effects of septal lesions in traditional aversive learning paradigms, such as passive and active avoidance tasks, and the effects of anti-anxiety (i.e., anxiolytic) drugs in the same paradigms. His basic interpretation of these data is that the septum and related areas (e.g., hippocampus) act in concert in the modulation of fear or anxiety (Gray, 1982, 1991; Gray & McNaughton, 1983).

The results in support of this hypothesis, however, have not always been consistent or easy to interpret, perhaps owing to complications associated with the learning tasks themselves (Treit, 1990, Treit & Menard, in press). For example, the response requirements of traditional aversive learning paradigms (e.g., bar-pressing) are often incompatible with the animal's natural defensive responses (e.g., freezing). Consequently, laboratory avoidance learning often can be unreliable and difficult to establish (Bolles, 1970). In addition, measures used in these tests generally have not been shown to be selectively suppressed by drugs that suppress anxiety in humans (i.e., anxiolytics; see Davis, 1992; Treit, 1985a). Thus, it is not always clear that the noted changes in responding are a valid reflection of fear reduction. Furthermore, traditional aversive learning paradigms were specifically designed to study the "general laws of learning" rather than fear or anxiety *per se* (Treit, 1985a), making it difficult to separate treatment effects on fear or anxiety from effects on learning and memory (Cahill & McGaugh, 1990). Similarly, septal lesions can affect food and fluid consumption (e.g., Donovick, Burright, & Gittelson, 1969), making it difficult to separate lesion effects on anxiety from lesion

effects on appetite, in cases where punished responding is partly motivated by food or fluid rewards. Finally, fear or anxiety are usually inferred in traditional aversive learning paradigms from a unidirectional change in behaviour (e.g., conditioned suppression of responding). Therefore, nonspecific effects on general activity, response inhibition, or arousal can be easily mistaken for specific effects on fear or anxiety. Thus, for a variety of reasons, these traditional aversive learning paradigms may not be the most appropriate means for studying the neuroanatomical correlates of anxiety.

A simpler approach is to investigate the neuroanatomical correlates of anxiety using behavioral paradigms that are specifically designed to elicit animals' untrained reactions to aversive or threatening stimuli (i.e., "ethological" models; Cooper & Hendrie, 1994; Treit, 1985a). Although a number of these ethological models have been recently developed, two, the elevated plus-maze and the shock-probe burying tests, have received extensive behavioural, physiological, and pharmacological validation (reviewed below) and have been effectively used to study the role of the septal nucleus in anxiety (e.g., Menard & Treit, 1996a,b, 1997; Pesold & Treit; 1992, 1994, 1995, 1996; Treit & Pesold, 1990; Treit, Aujla, & Menard, 1998; Treit, Pesold, & Rotzinger, 1993a; for a review see Treit & Menard, in press).

The remainder of this introductory chapter provides a brief description of the elevated plus-maze and shock-probe burying tests, followed by a summary of evidence implicating the septum in the control of anxiety. As will become apparent, the septal nucleus seems to play an important, *excitatory* role in the modulation of anxiety. In particular, evidence indicates that activity in the septal nucleus is critical to rats' anxiety-

related responses in the elevated plus-maze and shock-probe burying tests. However, there is a paucity of research regarding the relative contribution of the septum and the hippocampus to rats' innate or untrained fear reactions. The absence of such data may be critical, as evidence (reviewed below) increasingly suggests that specific regions of the brain are differentially involved in the control of different fear reactions.

Behavioural, physiological and pharmacological validation of the elevated plus-maze

In the elevated plus-maze test, rats are allowed to freely explore a novel, elevated maze in which two opposite arms are open and two are enclosed with walls. During the 5-min test, rats normally avoid the open arms, restricting most of their activity to the enclosed arms . When rats are forced to stay in the open arms, they show increased physiological and behavioural signs of stress, e.g., increased plasma corticosterone, freezing behaviour and defecation (Pellow et al., 1985). Rats' avoidance of the open-arm does not habituate, and, in fact, increases with repeated testing (e.g., Treit, Menard, & Royan, 1993), suggesting it is an innate, defense response of evolutionary significance (Petrinovich, 1973). Further investigation into the precise source of aversion in the maze indicated that rats' fear of the open-arms is driven by thigmotaxis (Treit, Menard, & Royan, 1993), a natural defensive response in which rats remain close to vertical surfaces, perhaps shielding themselves from potential threats (e.g., avian predators; Barnett, 1966; Grossen & Kelly, 1973).

Open-arm avoidance is the major index of anxiety in this test (Pellow et al., 1985), although more recently, other indexes have been proposed and validated (e.g., Rodgers &

Johnson, 1995). Rats' open-arm avoidance is specifically suppressed by drugs that reduce anxiety in humans (e.g., benzodiazepine anxiolytics such as diazepam [Valium®]), and exacerbated by drugs that induce anxiety in humans ("anxiogenic" drugs such as yohimbine; Pellow & File, 1986). Furthermore, the test is relatively insensitive to drugs from other therapeutic classes (e.g., neuroleptics) that are not useful in the treatment of human anxiety (Lister, 1987; Pellow et al., 1985). In summary, rats' avoidance of the open arms of the plus-maze provides a simple, pharmacologically valid measure of anxiety that does not obviously involve complex learning or memory mechanisms and does not depend on food or fluid motivation (for a review see Hogg, 1996).

Behavioural, physiological and pharmacological validation of the shock-probe burying test

In the shock-probe burying test, rats shocked once from a stationary, electrified probe attached to one wall of a plexiglass test chamber characteristically spray bedding material toward or over the probe, with forward, alternating thrusts of their forepaws (i.e., burying behavior; Pinel & Treit, 1978). Burying is a robust, reliable response that does not require complex training procedures (i.e., it occurs in most animals after only a single, shock-probe pairing) and is rarely seen in non-shocked laboratory rats (Terleki et al., 1979). The response is unambiguously directed toward the 'fearful' stimulus, e.g., rats shocked from one of two identical probes, mounted on opposite walls of the test chamber, selectively bury the probe from which they received shock (Pinel & Treit, 1978). Burying, is enduring, evident even after 20 days between the shock and subsequent testing (Heynen,

Sainsbury, & Montoya, 1989; Pinel & Treit, 1978) and can be elicited in rats previously deprived of all particulate matter in ontogeny (Pinel et al., 1989). Furthermore, there is evidence that burying is an adaptive response of rodents in the wild: ground squirrels will defend themselves from predatory snakes by spraying ground material directly at the snake, or by sealing themselves from an approaching snake by burying the entrance to their burrows (Tower & Cross, 1990). These and other data suggest that burying behavior represents an innate defensive response toward present dangers or threats (for reviews see Treit, 1985a, 1991, 1994; Treit, Menard, & Pesold, 1994).

Burying behaviour is suppressed by a number of standard anxiolytic drugs (e.g., diazepam) and enhanced by putative anxiogenic drugs, such as yohimbine (Tsuda, Yoshishige, & Tanaka, 1988; Treit, 1987). The suppression of burying by anxiolytic drugs does not appear to be secondary to behavioural sedation (Treit & Fundytus, 1988; Romer, Di Scala, & Sander, 1990), associative learning deficits (Blampied & Kirk, 1983), or analgesia (Treit 1985b), and can be reversed by benzodiazepine antagonists such as flumazenil (Treit, 1987). Furthermore, the effects of anxiolytics can be distinguished from those of several non-anxiolytic compounds (e.g., Treit et al., 1981). Anxiolytic drugs can also antagonize passive avoidance of the shock-probe, and shock-induced increases in corticosterone and adrenaline (Treit & Fundytus, 1988; De Boer et al., 1990; Treit, 1990). Finally, like the plus-maze test, the burying test does not involve food or fluid rewards or prior training of a response.

Together, these data suggest that the elevated plus-maze and shock-probe burying tests, which may measure rats' innate defense reactions to either potential (plus-maze) or

present (burying) dangers in their environment, may be particularly useful for investigating the neural mechanisms of anxiety. Furthermore, the combined use of these two tests is particularly advantageous. In the plus-maze test, the major index of anxiety is rats' passive avoidance of the open arms and in the burying test the major index of anxiety is rats' active burying of the shock-probe. An anxiolytic effect in the plus-maze test is primarily indicated by an *increase* in a specific behaviour (open-arm exploration), whereas an anxiolytic effect in the burying test is primarily indicated by a *decrease* in a specific behaviour (probe-burying). Thus, anxiolytic profiles seen in *both* tests would be difficult to explain in terms of nonspecific effects on general activity, arousal, or behavioural inhibition. If the septum does play an excitatory role in anxiety, then interfering with septal activity (e.g., using lesion or intra-cerebral drug infusion techniques) should *increase* open arm exploration and *decrease* burying behavior. A number of tests of these predictions are described in the following sections.

The role of the septal nucleus in anxiety

In an initial study, rats given bilateral electrolytic lesions of the entire septum were tested in the two behavioural paradigms after 8 days of post-surgical recovery (Treit & Pesold, 1990). Septal lesions produced a clear, anxiolytic effect in the elevated plus-maze, selectively increasing both the percentage of entries rats made into the open arms and the percentage of time rats spent in the open arms (Treit & Pesold, 1990). In the shock-probe burying test, sham-lesioned controls showed substantial burying behaviour toward the shock probe, whereas not one of the septal-lesioned rats showed this response. These

latter results replicate those found in an earlier study (Gray et al., 1981). Although there was some initial post-operative hyper-reactivity (i.e., "septal rage") in septal-lesioned rats, this dissipated with handling, so that handling reactivity, shock reactivity and general activity levels were not different between septal- and sham-lesioned rats at the time of the tests. In short, it appeared that, similar to anxiolytic drugs, septal lesions selectively suppress anxiety reactions in rats.

Importantly, the bidirectional pattern of results obtained in the two tests after septal lesions challenges a number of counter-interpretations, including a general impairment in response inhibition. Such a deficit might produce anxiolytic-like effects in one test (impaired open-arm avoidance), but not in both tests (impaired burying behavior). Although it could be argued that both effects were due to spatial learning or memory deficits (e.g., Hagan et al., 1988), this account is inconsistent with septal-lesioned rats' unimpaired avoidance of the shock-probe.

Thus, these first experiments suggested that the septum plays an important excitatory role in the expression of anxiety in the plus-maze and shock-probe tests. However, it was not clear whether destruction of septal nuclei had actually produced these effects, since electrolytic lesions also destroy fibers of passage. Accordingly, a second series of experiments (Pesold & Treit, 1992) compared the effects of electrolytic lesions of the septum to the effects of excitotoxic lesions, which preferentially destroy cell bodies leaving fibers of passage intact. Both electrolytic and excitotoxic lesions of the septum (using kainic acid or quisqualic acid) produced comparable anxiolytic effects in the elevated plus-maze and the shock-probe burying test. These anxiolytic effects were not

associated with an increase in general activity, in the case of the plus-maze, or a decrease in shock-reactivity, in the case of the shock-probe burying test. Again, septal hyper-reactivity was not apparent at the time of the tests (8-11 days post-surgery). These results suggested that cells originating in the septum mediate anxiety-related behaviours in the plus-maze and burying tests.

A third series of experiments examined the anatomical specificity of the anxiolytic effects of septal lesions. Although earlier studies (Treit & Pesold, 1990; Pesold & Treit, 1992) had suggested that these anxiolytic effects occur after posterior septal lesions but not after anterior septal lesions, their anatomical specificity had not been clearly delineated with respect to classical subdivisions of the septum, such as the lateral and medial nuclei (Swanson & Cowan, 1979). Accordingly, the effect of electrolytic lesions of the lateral or medial septal on rats' anxiety reactions in the plus-maze and shock-probe tests were compared (Menard & Treit, 1996a). Lateral and medial septal lesions produced equivalent anxiolytic effects in both tests. In addition, similar anxiolytic effects were evident when lesions included septal areas just anterior to the fornix (dorsolateral septum) but not when lesions were restricted to septal areas just posterior to the fornix (i.e., the septofimbrial and triangular septal nuclei or "postcommissural" septum; Menard & Treit, 1996a). Taken together with previous results, and with results showing that anxiolytic effects did not occur after lesions to the "ventral septum" (i.e., BNST; Treit, Aujla, & Menard, 1998), these data suggest that classical subdivisions of the septum bounded rostrally by the genu of the corpus callosum, caudally by the fornix, and ventrally by the anterior commissure, play an exclusively excitatory role in the control of anxiety, as expressed in the plus-maze

and shock-probe tests.

A final series of experiments examined the role of the septum in mediating the anxiolytic actions of midazolam, a benzodiazepine-type anxiolytic. Benzodiazepines are thought to produce their pharmacological effects by binding to the benzodiazepine recognition site on the $GABA_A$ receptor complex , enhancing the affinity of the $GABA_A$ receptor for its neurotransmitter, and thereby facilitating the inhibitory activity of GABA (Zorumski & Isenber, 1991). In this way, stimulation of the $GABA_A$ receptor complex with midazolam (an "agonist") should be functionally similar to inhibition of septal activity using septal lesions. Therefore, infusion of benzodiazepine agonists into the septum should produce anxiolytic effects similar to those produced by septal lesions.

Indeed, local application of midazolam (10 μg) into the septal nucleus increased open-arm activity in the plus-maze test and decreased burying behaviour in the shock-probe burying test (Pesold & Treit, 1994). Importantly, co-infusions (15 μg) of the benzodiazepine receptor antagonist Ro 15-1788 (flumazenil) blocked these specific, anti-fear effects without producing any intrinsic activity by itself, suggesting that the actions of midazolam were mediated at the benzodiazepine receptor. In a subsequent experiment (Pesold & Treit, 1996), infusions of midazolam into the lateral septum were shown to selectively increase open-arm exploration and eliminate burying behaviour, whereas infusions of midazolam into the medial septal region produced neither of these anxiolytic effects. The anxiolytic effects of midazolam in the lateral septum were also blocked by co-administration of flumazenil. Thus, it appeared that benzodiazepine receptors in the lateral septum contribute to the anxiolytic actions of midazolam, whereas those in the medial

septum do not (Pesold & Treit, 1996). Consistent with the latter possibility, infusions of the benzodiazepine agonist chlordiazepoxide into the medial septum (which selectively impaired rats' spatial learning) did not alter their fear behaviors in an open field test (McNamara & Skelton, 1993).

At the same time, these infusion data are difficult to reconcile with previous lesion data, which showed clear anxiolytic effects after medial septal lesions (Menard & Treit, 1996a). These inconsistencies underline the need for caution when interpreting the roles of specific septal sub-nuclei in the control of anxiety. In retrospect, it may be that medial septal lesions disrupted critical fibers projecting to or from the lateral septum, producing an "anxiolytic" effect. Micro-infusions of midazolam into the medial septum would leave these critical fibers intact, allowing normal fear reactions in the plus-maze and shock-probe tests. Further studies are needed to confirm or dismiss these speculations.

At any rate, a number of observations from other laboratories concur with the contention that eliminating or suppressing activity in the septal nucleus *reduces* anxiety. For example, septal lesion-induced increases in open-arm activity have been replicated in other laboratories (Decker et al., 1998; Thomas & Snellman, 1996). Septal lesions have been shown to retard the acquisition of passive avoidance responding in the "conflict" test of anxiety; i.e., rats' responding for reward was suppressed by electric foot-shock (Yadin et al., 1993). Some passive avoidance tasks, which resemble "conflict" tests in their essential features, are suppressed by septal lesions (Hamilton, Kelsey, & Grossman, 1970). Neurotoxic lesions of the septum, using 5,7-DHT, have produced anxiolytic effects in the "social interaction test," which is another, well-validated ethological, animal model of

anxiety (Clarke & File, 1982). Finally, pharmacological suppression of the septum, induced by local application of the $GABA_A$ receptor agonist muscimol, has also been reported to produce anxiolytic effects in the Vogel conflict test (Drugan et al., 1986).

The relative role of the septum and other limbic structures in anxiety

At this point, one might be persuaded that the septum, a structure not widely associated with anxiety, may indeed play some role in its modulation. Nevertheless, the historical stature of the role of the septum in anxiety pales beside other limbic structures such as the amygdala (e.g., Davis, 1992; LeDoux, 1996). Thus, one question that arises naturally is the relative contribution of the septum in anxiety, compared to other limbic structures, such as the amygdala.

In order to investigate this issue, the effects of septal lesions were directly compared to those of amygdala lesions in the elevated plus-maze and shock-probe burying tests (Treit, Pesold & Rotzinger, 1993a). As in previous studies (e.g., Pesold & Treit, 1992), septal lesions selectively increased open-arm activity and decreased shock-probe burying, without altering rats' normal avoidance of the shock-probe. In contrast, amygdala lesions had no effects on open-arm avoidance or burying behaviour, but dramatically increased shock-probe contacts. This selective effect of amygdala lesions was not associated with changes in shock-reactivity or general activity levels and, thus, could not be attributed to non-specific effects (e.g., reduced shock-sensitivity or hyperactivity). The anxiolytic effect of amygdala lesions also did not appear to reflect a more general deficit in response inhibition, or passive avoidance, because amygdala-

lesioned rats avoided the open arms of the plus-maze to the same extent as sham-lesioned controls. Similarly, lesion-induced increases in probe contacts could not be attributed to an inability to associate the shock with the probe, because amygdaloid lesioned rats buried the probe to the same degree as sham-lesioned controls. This failure of amygdala lesions to significantly suppress burying behaviour in the shock-probe test has been independently replicated in three different laboratories (Roozendaal, Koolhaas, & Bohus, 1991; Kopchia, Altman, & Commissaris, 1992; Treit & Menard; 1997; Treit, Pesold, & Rotzinger, 1993b, Treit, Aujla, & Menard, 1998). Thus, it appeared that the amygdala and septum independently control the expression of different fear responses.

The results of subsequent infusion studies reinforced this general conclusion. Although infusions of midazolam (10 μg) into the septal nucleus increased open-arm activity in the plus-maze test and decreased burying behavior in the shock-probe test, infusions of midazolam into the amygdala (10 μg/side) produced neither of these anxiolytic effects (Pesold & Treit, 1994). Intra-amygdala midazolam did, however, seriously impair rats' shock-probe avoidance, an anxiolytic effect not produced by intra-septal midazolam. Co-infusions of flumazenil blocked each of these specific, anti-fear effects without producing any intrinsic activity by itself. These results suggested that benzodiazepine receptor systems within the amygdala and the septum differentially regulate specific fear-reactions.

Although the above study was not designed to investigate the effect of midazolam in discrete sub-nuclei of the amygdala, histological analysis revealed that the majority of cannulae were situated in the central amygdala. A subsequent study showed that rats'

passive avoidance of the shock-probe seemed to be influenced by activity in the central (but not basolateral) amygdala (Pesold & Treit, 1995). Specifically, bilateral infusions of midazolam (5 μg) into the central amygdala selectively increased the number of contact-induced shocks from the probe but did not affect either burying behaviour or open-arm avoidance, whereas this same treatment in the basolateral amygdala did not affect shock-probe avoidance or burying but did, surprisingly, produce a selective suppression of open-arm avoidance. It remains to be determined why lesions of the entire amygdala consistently lacked effects in the plus-maze (see above), when inhibiting activity in a smaller, sub-region of the amygdala produced clear anxiolytic effects in the same test. At any rate, anxiolysis in the plus-maze has been observed in a number of laboratories following local application of midazolam into the basolateral (but not central) amygdala (Green & Vale, 1992; Mesches, Bianchin, & McGaugh, 1996; Zangrossi Jr. & Graeff, 1994, but see Gonzalez, Andrews, & File, 1996). These infusion data further extend and refine the general conclusions derived from the lesion data; i.e., specific regions (or sub-regions) of the brain seem to exert distinct control over some fear reactions (probe-avoidance and burying), yet share common control over others (i.e., midazolm in either the septum or basolateral amygdala impairs open-arm avoidance).

A similar conclusion emerges when considering the comparative roles of the amygdala and hippocampus in fear regulation. In one study, rats with excitotoxic lesions of the hippocampus or the amygdala were exposed to pairings of an auditory cue and electric shock in one side of a distinctive two-compartment test chamber (Seldon et al., 1991). During subsequent testing, hippocampal lesioned rats were severely impaired in

choosing the "safe" side of the two-compartment chamber but showed normal suppression of drinking (in a separate test chamber) when the auditory cue was introduced (i.e., conditioned suppression of drinking). Conversely, amygdala lesioned rats were impaired in the conditioned suppression of drinking test, but showed normal preference for the "safe" side of the two-compartment chamber. Other studies have shown both associations and dissociations between the effects of hippocampal or amygdala lesions. For example, freezing induced by re-exposing rats to a test chamber previously paired with foot-shock (i.e., conditioned freezing) was suppressed by either amygdala or hippocampal lesions, whereas the potentiated acoustic startle response observed in the same test chamber (i.e., fear-potentiated startle) was suppressed by amygdala, but not hippocampal, lesions (McNish et al., 1997).

Very few studies have directly compared the role of the septum and hippocampus in anxiety using pharmacologically validated animal models of anxiety. This lacuna is surprising in light of Gray's (1982, 1991) theory that these structures share common control over anxiety. According to this theory, one might predict commonalities between the effects of septal and hippocampal lesions. In a direct test of this hypotheses, however, it was shown that septal lesions increased open-arm exploration in the plus-maze and suppressed burying in the shock-probe burying test, whereas hippocampal lesions produced neither of these effects (Treit & Menard, 1997). Hippocampal lesions, on the other hand, increased the number on contacts rats made with the electrified probe, an anxiolytic effect not observed in septal lesioned rats. This double dissociation suggests that the septum and hippocampus might exert distinct control over different fear reactions.

Thesis rationale and objectives

The studies summarized above suggest that the neural system responsible for mediating anxiety is complex and multifaceted, each part specialized for processing a particular aspect of anxiety. What these specialized roles are and how they are carried out is not known at present. However, it is unlikely that these data can be explained in terms of a generalized, global reduction in anxiety (cf. Treit & Menard, 1997; Treit, Pesold, & Rotzinger, 1993a). At the very least, current neurobiological theories of fear or anxiety, which tend to center around a single brain region, such as the amygdala (e.g., Davis, 1992; LeDoux, 1996) or the 'septo-hippocampus' (e.g., Gray 1982, 1991) may no longer be tenable. An explanatory model based on parallel processing of different fear reactions, although far from parsimonious, may ultimately prove to be more plausible. That is, the neural mechanisms of anxiety may resemble the neural mechanisms of sensorimotor control: i.e., a complex system of functionally independent, distributed, parallel pathways. How these parallel lines or networks are configured in order to produce harmonious and adaptive defensive behaviour is an important question that demands direct, systematic comparisons of the role of different brain structures in different animal models of anxiety.

Accordingly, although each of the following studies addresses specific questions, they were all designed to further explore the relationship of the septum and the hippocampus in the modulation of rats' fear responding in the elevated plus-maze and shock-probe burying tests. The intra-cerebral drug infusion technique was utilized in each study. Although this technique is not without potential pitfalls (e.g., non-specific effects due to pH, osmolarity and drug diffusion; for further details see Greenshaw, 1998; Myers,

1974), it is perhaps the most powerful tool currently available for determining the contribution of specific receptor systems to the functional roles of a given brain structure. Indeed, use of the infusion technique has generated a wealth of information regarding different receptor systems in the amygdala and their specific role in anxiety (for a review see Menard & Treit, in press). However, the effects of pharmacological compounds in septum and hippocampus on fear responding have received relatively little attention, a gap in our knowledge that the current work hopes to narrow.

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Chapter 2

The septum and the hippocampus differentially mediate the anxiolytic effects of R(+)-8-OH-DPAT

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Introduction

Numerous clinical and preclinical studies have confirmed the anxiety-reducing properties of 5-HT_{1A} receptor-type anxiolytics, such as buspirone (De Vry et al., 1992; Hindmarch et al., 1992; Treit, 1991b). These agents act as agonists (or partial agonists) at 5-HT_{1A} receptors located presynaptically, in midbrain raphe nuclei, and postsynaptically, in forebrain limbic structures (Palacios et al., 1990; Pazos & Palacios, 1985; Radja et al., 1991; Waeber & Moskowitz, 1995; Zifa & Fillion, 1992). Presynaptic activation of 5-HT_{1A} receptors reduces 5-HT cell firing, synthesis and release (Blier et al., 1989; de Montigny & Blier, 1992; Hjorth & Sharp, 1991; Hutson et al., 1989; Invernizzi et al., 1991; Sinton & Fallon, 1988; Sprouse & Aghajanian, 1987), while postsynaptic activation results in neuronal inhibition in limbic structures such as the septum and hippocampus (de Montigny & Blier, 1992; Leishman et al., 1994; Van den Hoof & Galvan, 1992; Zifa & Fillion, 1992).

Considerable attention has been focused on where the anxiolytic properties of 5-HT_{1A} agonists are mediated in brain. Direct application of 5-HT_{1A} agonists into presynaptic areas, such as dorsal or median raphe, consistently suppresses rodents' fear-responses in several animal models of anxiety, including the social interaction, elevated plus-maze, light/dark exploration, shock-induced ultrasonic vocalization, and Vogel-type conflict tests (Carli & Samanin, 1988; Carli et al., 1989; Costall et al., 1988; De Almeida et al., 1997; File & Gonzalez, 1996; File et al., 1996; Higgins et al., 1988, 1992; Hogg et al., 1994; Picazo et al., 1995; Remy et al., 1996; Schreiber & De Vry, 1993). At postsynaptic sites, application of 5-HT_{1A} agonists appears to have more complex effects.

Although high-dose infusions (e.g., 5 μg) of 5-HT_{1A} agonists into dorsal hippocampus (Jolas et al., 1995; Kataoka et al., 1991; Kostowski et al., 1989; Przegalinski et al., 1994; Schreiber & De Vry, 1993; Stefanski et al., 1993) or amygdala (Schreiber & De Vry, 1993) produced *anxiolytic* effects in the elevated plus-maze (Kostowski et al., 1989), ultrasonic vocalization (Jolas et al., 1995; Schreiber & De Vry, 1993), and conflict tests (Kataoka et al., 1991, Przegalinski et al., 1994; Schreiber & De Vry, 1993; Stefanski et al., 1993), low dose infusions (e.g., 0.1 μg) at the same sites produced *anxiogenic* effects in the social interaction test (Andrews et al., 1994; File et al., 1996; Gonzalez et al., 1996) and in a modified Geller conflict test (Hodges et al., 1987). Unfortunately, however, no single study has directly compared the effects of both low and high doses of 5-HT_{1A} agonists at selected postsynaptic sites using the same tests of anxiety. Therefore, it is not yet clear whether these complex, dose-specific drug effects are generalizable to other indices of anxiety or whether they are test- and/or site-specific.

With respect to site-specificity, it is curious that the effects of intra-septal 5-HT_{1A} agonists in animal models of anxiety have received so little attention (De Almeida et al., 1997). This is odd, given the fact that the septum contains high densities of post-synaptic 5-HT_{1A} receptors (Pazos & Palacios, 1985; Waeber & Moskowitz, 1995), and has been repeatedly implicated in the mediation of anxiety-related behaviors (Gray, 1982; Menard &Treit, 1996a,b; Pesold & Treit, 1992, 1994, 1996; Thomas, 1988; Treit, 1991a; Treit & Pesold, 1990; Treit et al., 1993a,b,c; Yadin et al., 1993).

A series of studies has shown that activity in the septum is critical for the expression of rats' fear reactions in the elevated plus-maze and the shock-probe burying

tests of anxiety. In the elevated plus-maze, rats normally avoid the two open arms of the maze and restrict most of their activity to the two closed arms (Pellow et al., 1985). In the shock-probe burying test, rats given bedding materials will "bury" a probe from which they have been shocked (Pinel & Treit, 1978; Treit et al., 1994). Anxiolytic drugs (e.g., diazepam) increase rats' open-arm exploration in the plus maze and decrease rats' burying response to the shock-probe (Pellow, 1986; Pellow et al., 1985; Treit, 1990), whereas "anxiogenic" drugs (e.g., yohimbine) decrease open-arm exploration and increase shock-probe burying (Pellow, 1986; Tsuda et al., 1988). Electrolytic or excitotoxic lesions of the septum produced anxiolytic-like effects in the plus-maze and shock-probe burying tests, i.e., open-arm exploration was increased and burying behavior was decreased (Menard & Treit, 1996a,b; Pesold & Treit, 1992; Treit, 1991a; Treit & Pesold, 1990; Treit et al., 1993a,b). These anxiolytic-like effects were not secondary to changes in general activity, handling reactivity or shock-reactivity. Furthermore, the same pattern of effects was produced when septal activity was inhibited via intra-septal infusions of the benzodiazepine-type anxiolytic, midazolam (Pesold & Treit, 1994).

Because septal activity is also inhibited by agonist activation of post-synaptic 5-HT_{1A} receptors (Leishman et al., 1994; Van den Hooff & Galvan, 1992), a reasonable expectation is that intra-septal infusions of a 5-HT_{1A} agonist should also increase open-arm exploration and decrease shock-probe burying. Accordingly, in Experiment 1 both high (5 and 10 µg) and low doses (0.25 µg) of R(+)-8-hydroxy-2-(di-n-propyl-amino)tertralin (R(+)-8-OH-DPAT) were infused into rat septum. The R(+) enantiomer is a full agonist at 5-HT_{1A} receptors, whereas the S(-) enantiomer is a partial agonist

(Hadrava et al., 1996; Yu et al., 1993). In Experiment 2, the possible interactions of infusion dose with infusion site were examined by directly comparing the effects of high and low doses of R(+)-8-OH-DPAT in the septum and the hippocampus.

Methods

Subjects

Subjects were naive, male Sprague Dawley rats (Charles River, Canada) weighing 250-300 g at the time of surgery. Following surgery, the rats were returned to the animal colony where they were individually housed in polycarbonate cages for the duration of the experiment. A 12 hour light:dark cycle was in effect (lights on at 0700), with food and water available ad lib.

Surgery and histology

Rats were anaesthetized with sodium pentobarbital (40 mg/kg i.p.) and ketamine hydrochloride (80 mg/kg i.m.) and placed in a Kopf stereotaxic instrument. Using flat skull coordinates (Paxinos & Watson, 1986), a 26-gauge stainless steel guide cannula (Plastic Products) was implanted either 1 mm above the septum (0.5 mm anterior and 0.4 mm lateral to bregma, 3 mm ventral to dura, with the cannula guide angled 4° towards the midline) or bilaterally, 1 mm above the dorsal hippocampus (3.1 mm posterior and ±2.6 mm lateral to bregma, 2.2 mm ventral to dura with the cannulae angled 20° toward the midline). These coordinates were established on the basis of previous work (Pesold &Treit, 1994) and pilot studies in this laboratory. Guide cannulae were secured to the

skull using jeweler's screws and dental cement, and the patency of the cannulae was maintained by the insertion of a stylet (Plastic Products). At the end of behavioral testing, rats were sacrificed with an overdose of chloral hydrate. Following intracardial perfusions of 10% formalin, a 0.3 µl infusion of Indian Ink was administered, in order to mark the location of the cannula tip. Brains were extracted, frozen and cut into 40 µm sections, using a freezing cryostat. Sections were dry-mounted onto gelatine-coated slides, and stained with thionine. The location of the cannulae tips was determined by an observer who was unaware of the corresponding behavioral data.

Behavioral testing

Elevated plus-maze.

The elevated plus-maze was a wooden, cross-shaped maze, with two opposing open arms (50 x 10 cm) and two opposing enclosed arms (50 x 10 x 50 cm), each with an open roof. The maze was elevated to a height of 50 cm. The testing room was dimly lit at the time of testing. Eight min following their infusion, rats were placed individually in the center of the maze, facing a closed arm, and allowed 5 min of free exploration. The behavior of each rat was videotaped on closed-circuit television. In addition, an observer, sitting quietly 1 m from the maze, recorded: a) total time in the open arms, b) total time in closed arms, c) number of entries into open arms, and d) number of entries into closed arms. An entry was defined as all four paws in the arm. The maze was cleaned with water after each rat was tested.

For the purpose of analysis, open-arm activity was quantified as a) time spent in

the open arms relative to the total time spent in the maze (open/total x 100) and b) number of entries into open arms relative to the total number of entries into any arm (open/total x 100). Anxiety-reduction in this test is typically indicated by an increase in the percentage of open-arm entries and an increase in the percentage of open-arm time. The number of closed-arm entries was also analyzed as a measure of general activity (for details see Pellow et al., 1985; Rodgers & Johnson, 1995).

Shock-probe burying.

The shock-probe burying apparatus was in a separate testing room. It consisted of a 40 x 30 x 40 cm plexiglass chamber having a 5 cm layer of bedding material (i.e., cat litter) spread evenly about its floor. On one wall of the chamber was a small hole (centered 2 cm above the bedding material) through which a 6 x 0.5 x 0.5 cm wire-wrapped plexiglass probe could be inserted. Electric current was delivered through the two copper wires wrapped around the probe. Shock intensity was adjusted with a variable resistor in series with a 2000 V shock source, and was set at 2 mA. Rats were individually habituated to the test chamber (without the shock-probe present) for 15 min on each of 4 consecutive days. On day 5, the shock-probe was inserted 6 cm into the test chamber and secured in place. Eight min following their infusion, rats were placed individually into the chamber. Whenever the rat touched the constantly electrified probe, with either its snout or a forepaw, it received a brief, 2 mA shock. The 15 min test duration began immediately upon delivery of the first shock. At the end of each test, the chamber was cleaned of faeces and the bedding material was smoothed to a uniform thickness of 5 cm.

The behavior of each rat was videotaped on closed circuit television, and the videotapes were analyzed by an observer who was unaware of the rats' treatment. The following behaviors were measured: a) total duration of time spent spraying bedding material toward the shock-probe via rapid, alternating, forward thrusts of the forelimbs (i.e., burying behavior), b) total number of shocks received, and c) total duration of immobility (e.g., standing still or lying on the chamber floor with no movement). In addition, rats' behavioral reaction to each shock was scored according to the following four point scale: 1) flinch involving only head or forepaw, 2) whole body flinch, with or without ambulation away from the probe, 3) whole body flinch, and/or jumping, followed by ambulation away from the probe, and 4) whole body flinch and jump (all 4 feet in the air), followed by running to the opposite end of the chamber. A mean shock reactivity score was derived for each rat by summing its shock reactivity scores and dividing by its total number of shocks. Anxiety-reduction in this test is indicated by a decrease in the duration of time spent burying the probe, without concomitant changes in general activity or shock-reactivity (for details see Treit et al., 1994).

Infusion Procedures and Drug Regimens

R(+)-8-OH-DPAT hydrobromide (Research Biochemicals International) was dissolved in 0.9% saline. Rats were gently hand-held and a 33-gauge stainless steel internal cannula (Plastic Products) was lowered to 1 mm below the tip of the guide cannula. The internal cannula was connected, via polyethylene tubing (PE-50) to a constant rate Hamilton microsyringe. Rats with septal implants were infused with 1 µl of

solution and those with hippocampal implants were infused with 1 µl/side. An infusion rate of 1 µl/min was used and the internal cannula was left in place for an additional 1 min to allow diffusion away from the tip. The displacement of a bubble inside the polyethylene tubing was monitored to confirm drug flow. In addition, prior to replacing the stylet, the top of the cannula guide was inspected for fluid efflux.

Rats were randomly assigned to their respective treatment groups, with different rats being used in each dose condition in each experiment. Rats were first tested in the plus-maze apparatus, and then in the shock-probe burying apparatus, with an inter-test interval of ten days. Previous work has shown no evidence of drug carryover effects at this inter-test interval (Treit et al., 1993c). Some rats were not tested in both paradigms due to displaced cannula mounts, post-infusion fluid efflux from the top of the cannula guide, or failure to contact the electrified probe in the burying test. In addition, only animals with verified cannula placements (see Figure 2-1) are represented in the sample sizes below.

Experiment 1: Prior to the elevated plus-maze test, rats received intra-septal infusions of either 0.9% saline (n = 10), 0.25 µg (n = 9), 5 µg (n = 12) or 10 µg (n = 9) of R(+)-8-OH-DPAT. Prior to the shock-probe burying test, rats received intra-septal infusions of either 0.9% saline (n = 9), 0.25 µg (n = 8), 5 µg (n = 10) or 10 µg (n = 8) of R(+)-8-OH-DPAT.

Experiment 2: One set of rats received infusions into the septum prior to the two tests [elevated plus-maze test: 0.9 % saline (n = 11), 0.1 µg (n = 9) or 10 µg (n = 9) of R(+)-8-OH-DPAT; shock-probe burying test: 0.9% saline (n = 10), 0.1 µg (n = 9) or 10

μg (n = 8) of R(+)-8-OH-DPAT]. A second set of rats received bilateral infusions into the dorsal hippocampus prior to the two tests [elevated plus-maze test: 0.9% saline (n = 9), 0.1 μg/side (n = 8) or 5 μg/side (n = 8) of R(+)-8-OH-DPAT; shock-probe burying test: 0.9% saline (n = 9), 0.1 μg/side (n = 9) or 5 μg/side (n = 9) of R(+)-8-OH-DPAT].

Statistical Analysis

Results were analyzed using one-way analyses of variance (ANOVAs), which, when significant, were followed by pairwise comparisons of group means using Duncan's test ($\alpha = 0.05$). In order to correct for non-normality and heterogeneity of variance, the duration of burying scores were transformed (natural log) prior to the ANOVA.

Results

Experiment 1: Effects of intra-septal R(+)-8-OH-DPAT

Elevated plus-maze. Direct application of R(+)-8-OH-DPAT (0.25, 5 or 10 ug) into the septum did not alter open-arm activity or general activity in the elevated plus-maze; ANOVA [**percent open-arm entries**: $F(3,36) = 0.23$, $p > 0.87$; **percent open-arm time**: $F(3,36) = 0.14$, $p > 0.92$; **closed-arm entries**: $F(3,36) = 2.34$, $p > 0.09$; see Table 2-1].

Shock-probe burying test. In contrast to its lack of effect in the elevated plus-maze, infusion of R(+)-8-OH-DPAT into the septum produced a dose-related reduction in burying toward the electrified shock-probe (see Figure 2-2). Significant between groups ANOVA [**burying**: $F(3,31) = 4.12$, $p < 0.01$] and subsequent pairwise comparisons

(Duncan's test, α =0.05) confirmed that rats infused with R(+)-8-OH-DPAT (5 and 10 µg) buried the shock-probe significantly less than did rats infused with either saline or 0.25 µg of R(+)-8-OH-DPAT. The latter two groups did not differ from each other. This reduction in burying was selective because none of the groups differed on any other measure in this test; ANOVA [**shock reactivity**: $F(3,31) = 1.26$, $p > 0.30$; **number of shocks** $F(3,31) = 0.40$, $p > 0.76$; **immobility**: $F(3,31) = 1.38$, $p > 0.26$; see Table 2-1].

Experiment 2: Effects of intra-septal or intra-hippocampal R(+)-8-OH-DPAT

Elevated plus-maze. Similar to Experiment 1, rats' open-arm activity was not altered after intra-septal infusions of R(+)-8-OH-DPAT (0.1 or 10 µg). In contrast, substantial increases in open-arm activity were evident after intra-hippocampal infusions of R(+)-8-OH-DPAT (0.1 or 5µg/side; see Figures 2-3 and 2-4). Significant between groups ANOVA [**percent open-arm entries**: $F(5,48) = 3.47$, $p < 0.01$; **percent open-arm time**: $F(5,48) = 4.02$, $p < 0.005$] and subsequent pairwise comparisons (Duncan's test, $\alpha = 0.05$) confirmed that compared to their respective controls, rats infused with R(+)-8-OH-DPAT into the hippocampus (but not septum) had significantly greater open-arm activity (see Figures 2-3 and 2-4). This increase in open-arm activity was specific because there were no group differences on either measure of general activity; ANOVA [**closed-arm entries:** $F(5,48)=0.15$, $p > 0.97$; see Table 2-2]. These results suggest that the hippocampus (but not the septum) mediates anxiolytic properties of R(+)-8-OH-DPAT in the elevated plus-maze.

Shock-probe burying test. As in Experiment 1, intra-septal infusion of R(+)-8-OH-DPAT (10 ug) produced a substantial reduction in burying behavior in the shock-probe test. This particular anxiolytic effect did not occur after intra-hippocampal infusions of R(+)-8-OH-DPAT (see Figure 2-5). Significant between groups ANOVA [**burying**: $F(5,48) = 2.68$, $p < 0.03$] and subsequent pairwise comparisons (Duncan's test, $\alpha = 0.05$) confirmed that rats infused with R(+)-8-OH-DPAT (10 ug) into the septum buried the shock-probe significantly less than did rats infused with saline into the septum, whereas none of the other groups differed from each other. Furthermore, none of the control measures varied significantly between the groups, suggesting that the reduction in burying shown by the septal group was selective; ANOVA **[shock reactivity**: $F(5,48) = 1.84$, $p > 0.10$; **number of shocks** $F(5,48) = 1.31$, $p > 0.27$; **immobility**: $F(5,48) = 0.91$, $p > 0.48$, See Table 2-2]. These results suggest that the septum (but not the hippocampus) mediates anxiolytic properties of R(+)-8-OH-DPAT in the shock-probe burying test.

Discussion

Intra-septal infusions of R(+)-8-OH-DPAT did not alter rats' open-arm activity in the elevated plus-maze test, whereas the same treatment produced dramatic, dose-related reductions in rats' burying behavior in the shock-probe burying test. Conversely, intra-hippocampal infusions of R(+)-8-OH-DPAT produced substantial, dose-related increases in open-arm activity in the elevated plus-maze test, but did not alter rats' burying behavior in the shock-probe burying test. Although further work is needed to characterize in detail the receptor-specificity of these results, the behavioral dissociations described above

strongly suggest that 5-HT_{1A} receptor systems in the septum and hippocampus differentially control the expression of specific fear reactions in rats.

The finding that intra-septal R(+)-8-OH-DPAT clearly reduced burying but, just as clearly, left open-arm avoidance intact, was unexpected. It has been consistently shown that suppressing septal activity increases open-arm activity and decreases burying behavior (Menard & Treit, 1996a,b; Pesold & Treit, 1992, 1994; Treit & Pesold, 1990; Treit et al., 1993a,b), suggesting that the septum mediates rat anxiety in *both* tests (see Chapter 1). Septal lesion-induced increases in open-arm activity have been replicated in other laboratories (Decker et al., 1995; Thomas & Snellman, 1996), and prominent fos-like immunoreactivity has been observed in the septum after rats are exposed to various aversive stimuli, including exposure to the elevated plus-maze (Duncan et al., 1996).

Be this as it may, the suppressive effect of R(+)-8-OH-DPAT on burying found in the present study does appear to be a selective anxiolytic effect, and cannot be easily explained in terms of non-specific effects on general activity, shock-sensitivity, or associative memory. A number of observations support this view. First, there was no indication that intra-septal R(+)-8-OH-DPAT influenced general activity in either test because the groups did not differ with regard to duration of immobility in the shock-probe test or general activity in the plus-maze test (i.e., closed arm entries). Flat body posture, which is notable after systemic injections of (±)-8-OH-DPAT (Tricklebank, 1985) or R(+)-8-OH-DPAT (unpublished observations), was not observed following intra-septal R(+)-8-OH-DPAT, further suggesting that changes in motility were not responsible for the reduction in burying. Second, there was no evidence of drug-induced analgesia (i.e.,

no reductions in shock-reactivity) which could account for reductions in burying behavior. Septal-infused rats passively avoided the electrified shock-probe to the same extent as saline-infused controls. This observation also is not consistent with analgesia, since rats with reduced shock-sensitivity would not be expected to avoid the electrified probe as diligently as saline-infused controls. Finally, although infusions of (±)8-OH-DPAT (5 μg) into the septum have been reported to produce 'memory' deficits in a step-through passive avoidance task (Lee et al., 1992), memory deficits seem unlikely in the current study for the reason noted previously: saline- and drug-treated rats avoided the shock-probe to the same extent. In sum, the overall pattern of results strongly suggests that the reduction in burying seen after intra-septal infusions of R(+)-8-OH-DPAT was indeed due to reduction of anxiety.

By the same token, the increased open-arm activity seen after intra-dorsal hippocampal infusions of R(+)-8-OH-DPAT seemed specific to anxiety reduction, as it was not associated with changes in general activity. Furthermore, this result reinforces previous reports of anxiolysis following direct application of 5-HT_{1A} agonists into the dorsal hippocampus in a number of animal tests of anxiety, including the plus-maze (Kostowski et al., 1989), open-field (Stefanski et al., 1993), shock-induced ultrasonic vocalization (Jolas et al., 1995; Schreiber & De Vry, 1993) and Geller- and Vogel-type conflict tests (Kataoka et al., 1991; Przegalinski et al., 1994; Schreiber & De Vry, 1993; Stefanski et al., 1993). Nevertheless, despite this convergent evidence that dorsal hippocampus mediates the anxiolytic properties of 5-HT_{1A} agonists, rats' fear responding in the shock-probe burying test was not altered by intra-hippocampal R(+)-8-OH-DPAT in

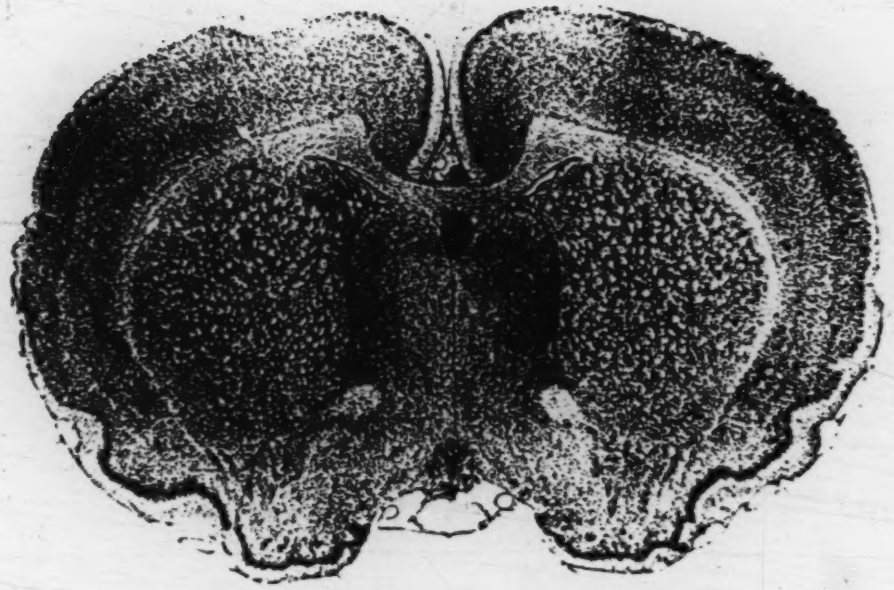
the present study.

In addition, there was no clear evidence for bidirectional effects of R(+)-8-OH-DPAT; i.e., anxiogenesis and anxiolysis after low- and high-dose infusions, respectively, in either the septum or dorsal hippocampus. Although low and high doses in dorsal hippocampus non-significantly increased and decreased mean duration of burying, these deviations were well within the range of variability normally seen in this test (Treit & Pesold, 1990; Treit et al., 1993a,b). Furthermore, intra-hippocampal application of R(+)-8-OH-DPAT produced significant, unidirectional increases in open-arm activity in the plus-maze (i.e., anxiolysis) at both low and high doses. Although a lower dose might have been anxiogenic in our tests, intra-hippocampal infusions of 0.05 or 0.1 μg of (±)8-OH-DPAT also failed to produce anxiogenesis in a previous study using the plus-maze test and, in fact, produced a non-significant trend towards anxiolysis at 0.2 μg (File et al., 1996). Furthermore, infusion of 0.1 μg of (±)8-OH-DPAT into dorsal hippocampus produced significant anxiolytic effects in an open-field test, and non-significant, anxiolytic-like increases in a shock-suppressed drinking test, which reached significance at 0.5 μg (Stefanski et al., 1993). Combined with the current results, these findings suggest that the anxiogenic effects of intra-dorsal hippocampal (±)8-OH-DPAT (0.1 μg), obtained by Andrews et al. (1994) and File et al. (1996), may be test specific. Although our results do not speak to whether this caveat extends to other limbic structures, the finding that intra-amygdaloid (±)8-OH-DPAT (0.1 ug) had anxiogenic effects in the social interaction test (Gonzalez et al., 1996) but not in the plus-maze test (Gonzalez et al., 1996; Zangrossi Jr. & Graeff, 1994) suggests that this may be the case.

Finally, an elegant argument has been made that anxiety-reduction following high-dose application of 5-HT_{1A} agonists into limbic structures may be due to diffusion of the compound back to raphe nuclei (De Vry, 1995; Jolas et al., 1995; Remy et al., 1996). However, diffusion would not account for the double dissociation obtained in the current study: i.e., high doses of R(+)-8-OH-DPAT reduced burying but not open-arm avoidance when infused into the septum and, conversely, reduced open-arm avoidance but not burying when infused into the hippocampus. Furthermore, in other experiments, intra-amygdaloid infusions of R(+)-8-OH-DPAT (5 ug/side) did not affect burying in the shock-probe burying test, whereas they did significantly increase the number of contacts made with the electrified shock-probe (Treit & Menard, 1996). This passive avoidance deficit was not seen in the current study after intra-septal or intra-hippocampal infusions of R(+)-8-OH-DPAT.

In conclusion, the anxiolytic properties of R(+)-8-OH-DPAT are differentially mediated by the septum and dorsal hippocampus. These dissociations suggest that postsynaptic 5-HT_{1A} receptors in different limbic structures exert distinct control over different fear behaviors. Furthermore, these results suggest that septal cells expressing 5-HT_{1A} receptors may play a role in some (burying) but not all (open-arm avoidance) fear responses mediated by the septum.

Figure 2-1. Photomicrographs showing the location of the cannula tips for intra-septal (top) and intra-hippocampal (bottom) infusions of R(+)-8-OH-DPAT.



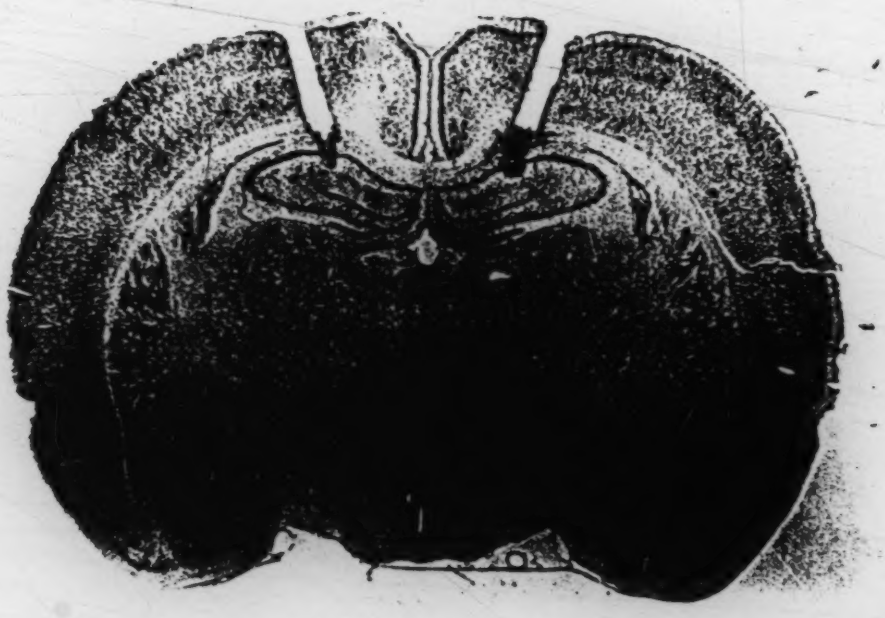


Table 2 - 1

Mean (± s.e.m.) activity and reactivity after intra-septal infusions of R(+)-8-OH-DPAT

	Dose (μg)			
Behavior	0.0	0.25	5.0	10
% Open-arm entries	22.64 (±5.32)	18.75 (±5.68)	23.28 (±4.97)	24.40 (±4.96)
% Open-arm time	16.19 (±6.39)	14.49 (±5.81)	19.83 (±4.63)	18.14 (±7.37)
Closed-arm entries	9.10 (±0.82)	7.11 (±0.81)	9.00 (±0.64)	6.67 (±1.05)
Shock-reactivity	2.33 (±0.24)	2.03 (±0.18)	2.49 (±0.26)	2.63 (±0.20)
Number of shocks	2.00 (±0.28)	2.00 (±0.27)	2.30 (±0.15)	2.38 (±0.50)
Immobility (s)	37.77 (±17.39)	11.38 (±5.58)	14.1 (±7.49)	43.5 (±20.25)

Figure 2-2. Mean (± s.e.m.) duration of burying (log s) after intra-septal infusions of R(+)-8-OH-DPAT (*$p < 0.05$ compared with controls).

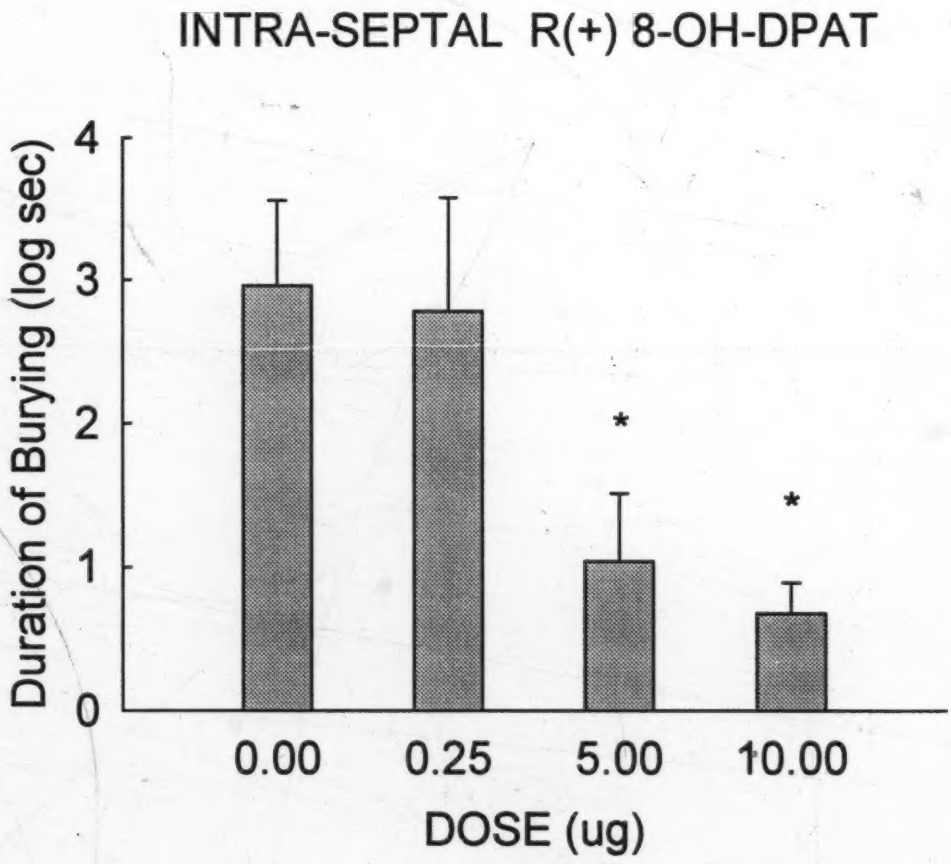

INTRA-SEPTAL R(+) 8-OH-DPAT
Duration of Burying (log sec)
4
3
2
1
0
*
*
0.00
0.25
5.00
10.00
DOSE (ug)

Figure 2-3. Mean (± s.e.m.) percentage of entries made into the open arms of the plus-maze after intra-septal (open bars) or intrahippocampal (shaded bars) infusions of R(+)-8-OH-DPAT (*$p < 0.05$ compared with controls).

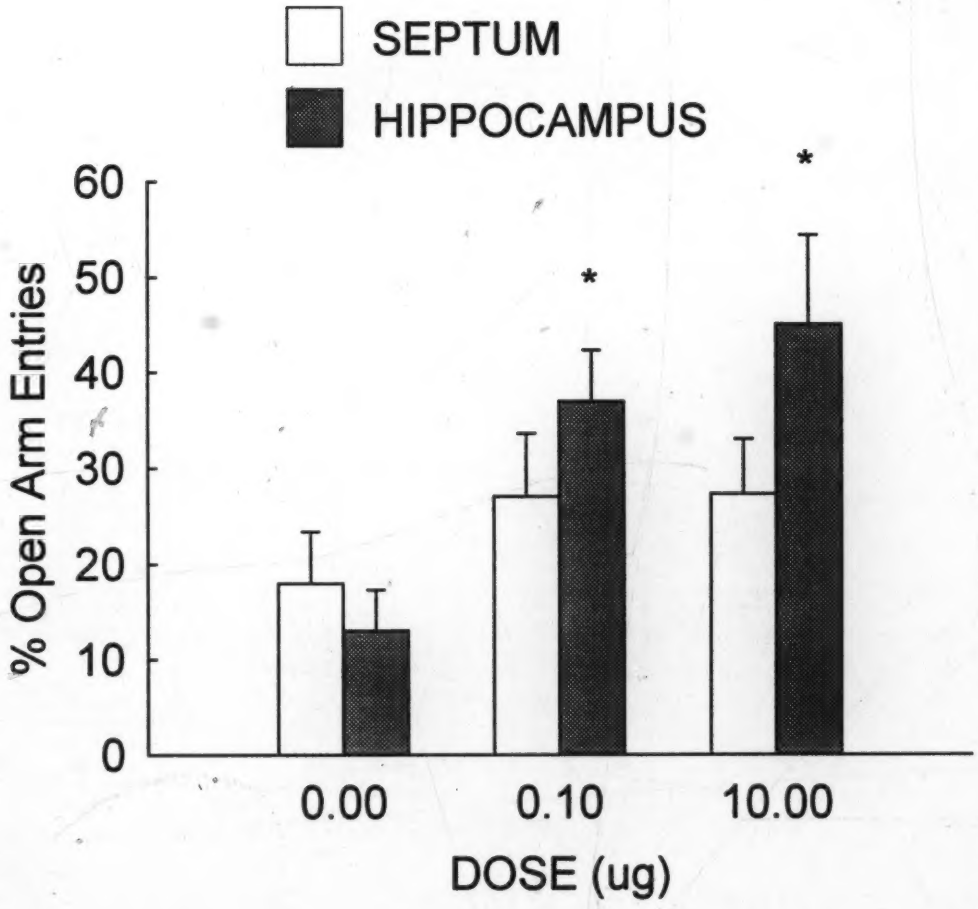

SEPTUM
HIPPOCAMPUS
% Open Arm Entries
60
50
40
30
20
10
0
*
*
0.00
0.10
10.00
DOSE (ug)

Figure 2-4. Mean (± s.e.m.) percentage of time spent in the open arms of the plus-maze after intra-septal (open bars) or intrahippocampal (shaded bars) infusions of R(+)-8-OH-DPAT (*$p < 0.05$ compared with controls).

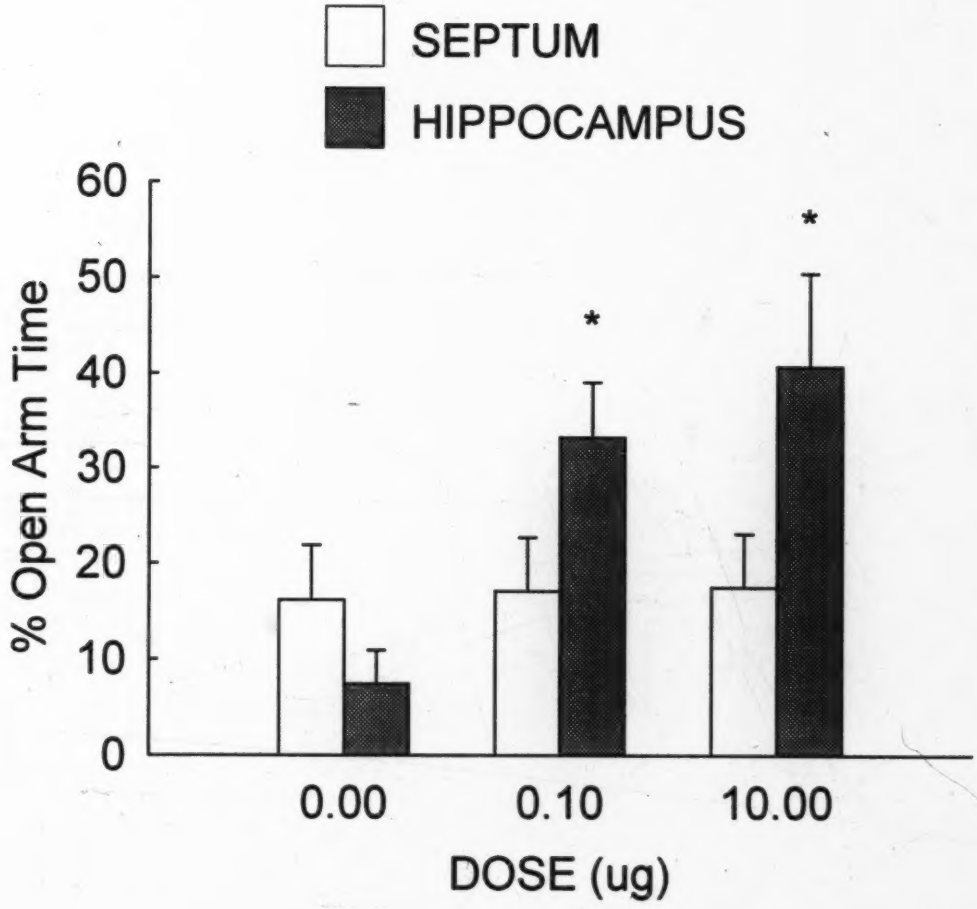
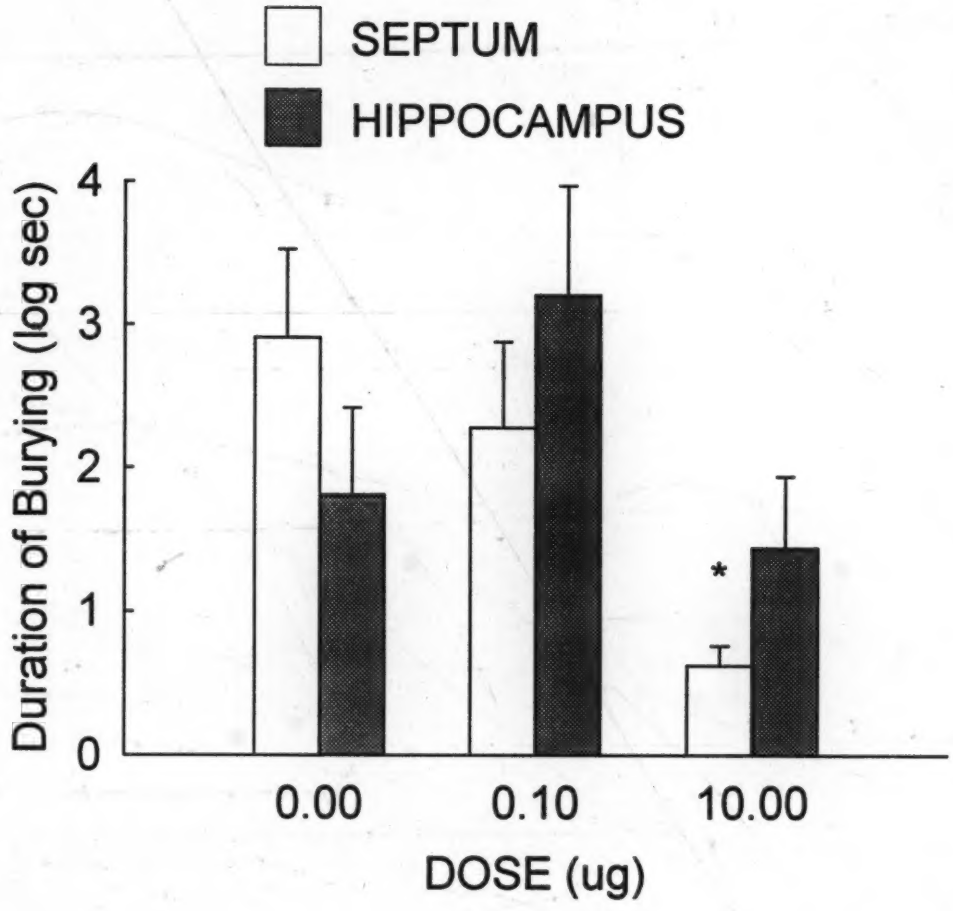

SEPTUM
HIPPOCAMPUS
% Open Arm Time
60
50
40
30
20
10
0
*
*
0.00
0.10
10.00
DOSE (ug)

Table 2 - 2

Mean (± s.e.m) activity and reactivity scores after intra-septal or intra-hippocampal infusions of R(+)-8-OH-DPAT (Experiment 2).

	Site					
	Intra-septal (μg)			Intra-hippocampal (μg/side)		
	Dose (μg)					
	0.0	0.1	10	0.0	0.1	5.0
Behavior						
Closed-arm entries	7.64 (±0.82)	7.33 (±0.76)	7.67 (±1.32)	8.11 (±1.21)	8.25 (±0.80)	7.25 (±1.16)
Shock-reactivity	2.26 (±0.15)	1.83 (±0.17)	1.89 (±0.25)	1.96 (±0.26)	2.19 (±0.19)	2.64 (±0.30)
Shock number	2.60 (±0.31)	2.22 (±0.28)	2.13 (±0.40)	2.44 (±0.34)	2.44 (±0.38)	1.56 (±0.24)
Immobility (s)	52.3 (±29.70)	14.11 (±5.89)	26.25 (±9.25)	52.00 (±24.97)	29.89 (±6.97)	15.44 (±2.50)

Figure 2-5. Mean (± s.e.m.) duration of burying (log s) after intra-septal (open bars) or intra-hippocampal (shaded bars) infusions of R(+)-8-OH-DPAT (*$p < 0.05$ compared with controls).


SEPTUM
HIPPOCAMPUS
Duration of Burying (log sec)
4
3
2
1
0
*
0.00
0.10
10.00
DOSE (ug)

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Chapter 3

Intra-septal infusions of excitatory amino acid receptor antagonists: Effects on different fear responses

Introduction

As reviewed in Chapter 1, the septum seems to play an important, excitatory role in the regulation of anxiety. Substantial support for this proposition comes from the finding that activity in the septal nucleus is critical for the expression of rats' fear reactions in the elevated plus-maze and shock-probe burying tests. Specifically, electrolytic or excitotoxic lesions of the septum have been shown to produce profound increases of open-arm exploration in the plus-maze and decreases in burying in the shock-probe burying test (for a review see Chapter 1). These anxiolytic-like effects were not secondary to changes in general activity, handling reactivity or shock reactivity. Furthermore, the same pattern of effects was produced when septal activity was inhibited via intra-septal infusions of the benzodiazepine-type anxiolytic, midazolam (Pesold & Treit, 1994, 1996). However, intra-septal infusions of the inhibitory, 5-HT1A receptor-type anxiolytic, R(+)-8-OH-DPAT produced clear, anxiolytic-like reductions in burying but left open-arm avoidance intact, suggesting that these two fear responses may be modulated by separate receptor systems within the septal nucleus (Chapter 2; Menard & Treit, 1998). Nevertheless, it seems clear that eliminating or inhibiting activity in the septal nucleus generally results in a reduction in fear responding. Conversely, we might expect that excitation of septal cells generates fear- or anxiety-related responses and, further, that blocking this excitation might result in anxiolysis.

As with the CNS in general, excitatory neurotransmission in the septal nucleus is primarily mediated at excitatory amino acid (EAA) receptors, such as NMDA and non-NMDA (i.e., AMPA and kainate) receptors (Kumamoto, 1997; Gallagher et al., 1995;

Ozawa, Kamiya & Tzuzuki, 1998). Both NMDA and non-NMDA receptors are highly expressed in the septum (Jacobson & Cotrell, 1993; Petralia & Wenthold, 1992; Rogers et al., 1991; Wisden & Seeburg, 1993). NMDA, AMPA and kainate receptors are ionotropic receptors, consisting of ligand-gated cation channels (for reviews see Ozawa et al., 1998; Kumamoto, 1997). Although the role of kainate receptors in excitatory synaptic transmission is not yet clear, it is known that AMPA receptors mediate fast excitatory post synaptic potentials (EPSPs), whereas NMDA receptors mediate a slower, more prolonged component of the EPSP (Ozawa et al., 1998). Furthermore, the NMDA channel is unique in that it is both ligand- and voltage-gated. Thus, not surprisingly, low frequency, low intensity focal stimulation of the primary EAA input fibres to the dorsolateral septum (i.e., fimbrial fibres from the hippocampus) leads to the activation of non-NMDA receptors, whereas higher frequency and/or higher intensity stimulation leads to the activation of both non-NMDA and NMDA receptors (e.g., Gallagher & Hasuo, 1989; for a review see Gallagher et al., 1995). Finally, NMDA channel activation also has an absolute requirement for glycine, which binds to a strychnine-insensitive glycine-binding site present on the NMDA channel (Ozawa et al., 1998). Given these differential properties of NMDA and non-NMDA receptors, we should not be surprised if their functional roles in regulating behavior also differ.

There is some initial evidence that NMDA receptors and non-NMDA receptors expressed in certain regions of the brain might play differential roles in the modulation of different fear behaviors. For example, infusions of NMDA receptor antagonists (e.g., AP-5) into the basolateral amygdala failed to alter fear responding in the fear-potentiated

startle test (Campeau, Miserendino, & Davis, 1992; Miserendino et al., 1990), whereas infusions of the non-NMDA receptor antagonist, CNQX at the same site dose-dependently blocked the expression of fear-potentiated startle (Kim et al., 1993). On the other hand, infusions of either NMDA or non-NMDA antagonists (AP-5 and CNQX, respectively) into the basolateral amygdala had selective anxiolytic effects in the social interaction test (Sajdyk & Shekhar, 1997a,b).

If NMDA and non-NMDA receptors in the septal nucleus play similar roles in regulating fear behaviors, then intra-septal infusions of either AP-5 or CNQX should increase open-arm exploration and decrease burying behavior. If, however, these receptors play more specific roles in the regulation of different fear behaviors, then dissociations of this pattern might emerge.

EXPERIMENT 1

Methods

Subjects

Subjects were 45 naive, male Sprague Dawley rats (Charles River Canada) weighing between 250-290 g at the time of surgery. Following surgery, the rats were returned to the animal colony where they were maintained in the same manner as outlined in Chapter 2.

Surgery and histology

Anesthetized rats were implanted with a single guide cannula aimed 1 mm above

the septal nucleus. All surgical methods (including atlas coordinates) and histological methods were the same as in Chapter 2.

Behavioral testing

Behavioral testing was the same as in Chapter 2, with the following exceptions. Following 7 days of post-surgical recovery, rats were briefly handled on each of 4 consecutive days. On the following day, rats received their first infusion 5 min prior to being tested in the elevated plus-maze. Eight days later, rats received their second infusion 5 min prior to being tested in the shock-probe burying test.

Infusion procedures and drug regimens

Infusion procedures were the same as in Chapter 2. AP-5 (RBI) was dissolved in 0.9% saline, whereas CNQX (RBI) was dissolved in distilled water. Drug doses were selected on the basis of pilot work and previous literature (Campeau, Miserendino & Davis, 1992; Kim et al., 1993). Rats were infused with 1 µl of solution at an infusion rate of 1 µl/min, with the internal cannula left in place for an additional min to allow diffusion away from the tip. Rats were randomly assigned to their respective treatment groups and received the same treatment prior to each test, except as noted below. Some rats were not tested in both paradigms due to displaced cannula mounts, post-infusion efflux from the end of the cannula guide or failure to contact the electrified probe in the burying test. The number of animals in each treatment group depicted below had verified cannula placements (Figure 3-1).

Before the elevated plus-maze test, the rats received intra-septal infusions of either 0.9 % saline (n = 10), 5 μg of CNQX (n = 9) or 5 μg of AP-5 (n = 10). Before the shock-probe burying test, the same rats received intra-septal infusions of either 0.9 % saline (n = 10), 5 μg of CNQX (n = 8) or 5 μg of AP-5 (n = 10). In order to rule out drug carry-over effects, an additional group of rats (n = 15) received intra-septal infusions of saline prior to a 5 min trial in the plus-maze, followed 8 days later by intra-septal infusions of either 0.9% saline (n = 4), 5 μg of CNQX (n = 6) or 5 μg of AP-5 (n = 5) just prior to the burying test. Because there was no evidence of carry-over effects for either drug on any measure taken in the shock-probe burying test (all p values > 0.15), data from this additional group of rats was included in the burying analysis, yielding final groups totals of 0.9% saline (n = 14), CNQX (n = 14) and AP-5 (n = 15).

Statistical analysis

Statistical analysis was the same as in Chapter 2. Specifically, results were analyzed using one-way analysis of variance (ANOVA) with subsequent pairwise comparisons (Duncan's test, $\alpha = 0.05$) where appropriate. In order to correct for non-normality and heterogeneity of variance, the duration of burying scores were transformed (natural log) prior to the ANOVA.

Results

Elevated plus-maze. Intra-septal infusions of CNQX (5 μg) dramatically increased rats' open-arm activity, whereas intra-septal infusion of AP-5 (5 μg) did not (Figure 3-2).

Significant between groups ANOVA [**percent open-arm entries**: $F(2,26) = 10.50$, $p < 0.001$; **percent open-arm time**: $F(2,26) = 18.19$, $p < 0.001$] and subsequent pairwise comparisons (Duncan's test, $\alpha = 0.05$) confirmed that rats infused with CNQX had significantly greater percentages of open-arm entries and open-arm time than did rats infused with either saline or AP-5, whereas these latter two groups did not differ from each other. Furthermore, there were no treatment effects on general activity [**closed-arm entries**; $F(2,26) = 0.86$, $p > 0.43$; see Table 3-1], confirming that the CNQX-induced increases in open-arm activity were specific to fear reduction. Thus, it appeared that non-NMDA receptors in the septum are involved in the expression of fear-related behaviors in the elevated plus-maze, whereas NMDA receptors are not.

Shock-probe burying test. Intra-septal infusions of either CNQX (5 μg) or AP-5 (5 μg) resulted in profound reductions in burying in the shock-probe burying test (Figure 3-3). Significant between groups ANOVA [**burying**: $F(2,40) = 6.50$, $p < 0.005$] and subsequent pairwise comparisons (Duncan's test, $\alpha = 0.05$) confirmed that rats infused with either CNQX or AP-5 did not differ from each other, whereas both drug-treated groups buried the shock-probe significantly less that did saline-treated controls. Although the general activity levels of AP-5 treated rats did not differ from the other groups, CNQX-treated rats spent significantly more time standing or lying still in the test chamber, compared to saline-treated rats [**immobility**: $F(2,40) = 3.43$, $p < 0.05$; Duncan's test, $\alpha = 0.05$; see Table 3-1]. This suggests some caution regarding the behavioral specificity of CNQX-induced reductions in burying. However, CNQX did not alter measures of general

activity in the plus-maze test (see Table 3-1), suggesting that CNQX does not produce a global impairment in motor functioning. At any rate, these data indicate that NMDA, and perhaps non-NMDA, receptor activation in the septal nucleus is critical for the expression of burying behavior.

Intra-septal AP-5 (5 μg), but not CNQX (5 μg), increased the number of contact-induced shocks rats received from the electrified probe; ANOVA [**number of shocks**: $F(2,40) = 3.43$, $p < 0.03$; see Figure 3-4]. Pairwise comparisons confirmed that AP-5-treated rats received significantly more shocks than did saline or CNQX-treated rats, whereas the latter groups did not differ (Duncan's test, $\alpha = 0.05$). Infusions of AP-5 also significantly increased shock reactivity scores, relative to infusions of saline or CNQX, which did not differ [**shock reactivity**: $F(2,40) = 5.21$, $p < 0.03$; Duncan's test, $\alpha = 0.05$; see Table 3-1]. Although this latter finding ruled out analgesia as a nonspecific effect, further analysis was conducted to determine whether shock reactivity and the number of shocks received were related. This possibility was ruled out by subsequent analysis of covariance (ANCOVA), using shock reactivity as the covariate; i.e., AP-5 induced increases in probe contacts remained significant following ANCOVA [**number of shocks**: $F(2,39) = 5.00$, $p < 0.01$]. Overall, these data suggest that NMDA, but not non-NMDA, receptors in the septum regulate rats' passive avoidance of the shock-probe.

EXPERIMENT 2

The finding that intra-septal infusions of AP-5 increased the number of contact-induced shocks in the shock-probe burying test was entirely unexpected. This is because

septal lesions or intra-septal infusions of inhibitory compounds, such as midazolam and R(+)-8-OH-DPAT consistently fail to impair rats' normal avoidance of the shock-probe, whereas the same treatments in the amygdala result in profound deficits in shock-probe avoidance (for a review see Chapter 1; Menard & Treit, in press). Furthermore, the magnitude of the effect of AP-5 on probe avoidance was small, relative to that produced by amygdala lesions; i.e., intra-septal AP-5 treated rats received approximately 2-3 probe contact-induced shocks, whereas amygdala-lesioned rats typically receive upwards of 6-8 shocks (Treit, Pesold, & Rotzinger, 1993a,b). Accordingly, it seemed important to further examine the reliability of the effects of intra-septal AP-5 on shock-probe avoidance.

Methods

Methods and materials were the same as in the preceding experiment (Experiment 1; Chapter 3), with the following exceptions. Eighteen anesthetized Sprague Dawley rats (Charles River Canada), weighing between 250-290 g, were surgically implanted with a single guide cannula aimed 1 mm above the septal nucleus, using flat-skull coordinates from Paxinos and Watson (1986); 0.7 mm anterior and 0.4 mm lateral to bregma, 3 mm ventral to dura with the cannula angled 4° towards the midline. After 8 days of post-surgical recovery, rats were briefly handled on each of 4 consecutive days. The next day, rats received intra-septal infusions of 0.9% saline (n = 18), 5 min prior to the elevated plus-maze test. Nine days later, the rats received intra-septal infusions of either 0.9% saline (n = 9) or 5 μg of AP-5 (n = 9) 5 min prior to the shock-probe burying test. Histological analysis revealed that 7 animals given saline and 8 animals given AP-5 had

appropriately placed implants (see Figure 3-5).

Results

Shock-probe burying test. Similar to Experiment 1, intra-septal infusions of AP-5 (5 μg) induced profound decreases in burying behavior and small, but significant increases in the number of probe contact-induced shocks (see Figure. 3-6); ANOVA [**burying**: $F(1,13) = 13.57$, $p < 0.01$; **number of shocks**: $F(1,13) = 9.20$, $p < 0.01$]. In contrast to the prior experiment, AP-5 and saline-treated rats did not differ on any other measure in this test; ANOVA [**shock reactivity**: $F(1,13) = 0.92$, $p > 0.35$; **immobility**: $F(1,13) = 0.46$, $p > 0.5$; see Table 3-2].

Discussion

Intra-septal infusions of the non-NMDA receptor antagonist, CNQX substantially suppressed open-arm avoidance in the plus-maze and burying behavior in the shock-probe burying test, but left rats' normal avoidance of the shock-probe intact. Conversely, intra-septal infusions of the NMDA receptor antagonist, AP-5 suppressed burying and shock-probe avoidance, but left rats' normal avoidance of the open arms intact. Although complex, these behavioral dissociations suggest that non-NMDA receptors and NMDA receptors within the septal nucleus have specific roles in the regulation of different fear reactions.

In light of the numerous demonstrations of increased open-arm exploration in septal-lesioned rats (see introduction), the anxiolytic actions of intra-septal infusions of

CNQX in the plus-maze were not unexpected. Furthermore, these CNQX-induced increases in open-arm activity seemed specific to fear reduction, because they were not accompanied by changes in general activity (i.e., closed-arm entries). On the other hand, the failure of intra-septal AP-5 to alter rats' open-arm avoidance was somewhat surprising, because direct application of either CNQX or AP-7 (another NMDA receptor antagonist) into the DPAG has been previously shown to produce anxiolysis in the plus-maze test (Guimaraes et al., 1991; Matheus & Guimaraes, 1997; Matheus et al., 1994). This raises the possibility that intra-septal AP-5 *did* reduce rats' fear in the plus-maze, but that this fear reduction was masked by non-specific effects on motor activity (e.g., sedation or ataxia). However, this possibility seems remote given the normal levels of general activity displayed by AP-5 treated rats. In short, the observed dissociation strongly suggests that non-NMDA (but not NMDA) receptors in the septal nucleus regulate rats' fear behaviors in the elevated plus-maze.

In the shock-probe burying test, intra-septal CNQX and AP-5 both produced a profound reduction in burying behavior, whereas AP-5 (but not CNQX) also suppressed rats' normal avoidance of the electrified probe. Non-specific effects on motor activity would not account for the drug-induced reductions in burying, because neither drug systematically altered measures of general activity (i.e., CNQX-treated rats displayed normal levels of general activity in the plus-maze, as did AP-5 treated rats in both tests). Similarly, drug-induced hyperactivity (which could increase 'accidental' contacts with the probe) would not account for the effect of AP-5 on shock-probe avoidance. Analgesic effects (which could reduce both probe-avoidance and burying behavior) seem unlikely in

the current study, because neither drug diminished rats' sensitivity to electric shock (i.e., mean reactivity scores were not reduced relative to controls). In fact, AP-5 treated rats in Experiment 1 displayed a heightened sensitivity to shock, which might be expected to increase shock-probe avoidance and burying behavior. In short, although clearly complex, the effects of intra-septal CNQX and AP-5 in the burying test are not easily explained by effects on general activity or pain sensitivity and, thus, might be suggestive of fear reduction.

Alternatively, NMDA and non-NMDA receptors have been heavily implicated in brain mechanisms underlying learning and memory (e.g., Maren, 1996). For example, direct application of CNQX or AP-5 into hippocampus or amygdala blocks the acquisiton of conditioned fear responses in a variety of tasks (Bianchin et al., 1996; Campeau et al., 1992; Fanselow & Kim, 1994; Kim & McGaugh, 1992; Mesches, Bianchin, & McGaugh, 1996; Miserendino et al., 1990; Izquierdo et al., 1997). Although similar studies have yet to be conducted in septum, it could be argued that the effects of intra-septal AP-5 and CNQX in the shock-probe burying test were secondary to a drug-induced deficit in associative learning. More specifically, if drug-treated rats failed to learn the relationship between the probe and the shock they would be unlikely to avoid further probe contacts or to engage in probe burying. However, this explanation is incompatable with the finding that saline- and CNQX-treated rats avoided the shock-probe to a similar degree. Furthermore, given the profound disruption in fear conditioning produced by similar doses of AP-5 in other regions of the brain (e.g., amygdala; Miserendino et al., 1990), it is curious that intra-septal AP-5 produced such small, albeit reliable, effects on shock-probe

avoidance. Thus, although a learning defict cannot, at present, be conclusively ruled out, the effects of intra-septal CNQX and AP-5 in the shock-probe burying test appear to be more indicative of fear reduction. This interpretation agrees with the selective anxiolytic effects observed in various animal tests of anxiety following local application of NMDA or non-NMDA antagonists into other brain structures (i.e., hippocampus, amygdala and periaqueductal grey; Guimaraes et al., 1991; Kim et al., 1993; Matheus & Guimaraes, 1997; Matheus et al., 1994; Mesches et al., 1996; Plaznik, Nazar, & Jessa, 1994; Sajdyk & Shekhar, 1997a,b; Walker & Davis, 1997).

Be this as it may, the finding that intra-septal AP-5 reduced rats' passive avoidance of the shock-probe is nonetheless perplexing, because this effect is *not* typically observed following ablation or pharmacological inhibition of the septal nucleus (for a review see Menard & Treit, in press). However, the lateral septum has direct, inhibitory connections to the amygdala, which appear to be GABAergic (for reviews see Risold & Swanson, 1997a,b). Local disinhibition of this pathway (e.g., by reducing excitation at septal GABAergic interneurons) might increase the release of GABA from the septum to the amygdala, which, in turn, might lead to an increase in shock-probe contacts. Indeed, local facilitation of the inhibitory actions of GABA in the amygdala via direct application of the BZ agonist, midazolam, has been shown to selectively increase the number of contact-induced shocks rats received from the probe (Pesold & Treit, 1994, 1996). Although speculative, the possibility that intra-septal AP-5 increased probe contacts by disinhibiting septal efferents to the amygdala would explain why septal-lesioned rats do not show similar increases in shock-probe contacts (i.e., septal lesions would remove inhibitory

input from the septum to the amygdala). Furthermore, local application of the NMDA antagonist, CPP into the septum has been shown to disinhibit septal projection neurons by reducing GABA outflow in the septal nucleus (Giovannini et al., 1994). Clearly, it would be both interesting and important to determine whether the effects of intra-septal AP-5 on shock-probe avoidance are reversed by co-infusions of the GABA agonist, muscimol.

Finally, the receptor specificity of the present results needs to be addressed. At high concentrations, CNQX has been shown to indirectly inhibit NMDA receptor actions through an antagonist action at the glycine receptor binding site on NMDA channels (Lester et al., 1989). Similarly, AP-5 has been shown to antagonize both NMDA and non-NMDA receptors (Honore et al., 1988). However, the behavioral dissociations obtained in the present study suggest that the observed effects of intra-septal CNQX and AP-5 were due to a selective antagonism of non-NMDA and NMDA receptors, respectively. Finally, because CNQX antagonizes both AMPA and kainate receptors (Honore et al., 1988), the specific identity of the non-NMDA receptor responsible the anxiolytic actions of intra-septal CNQX remains to be determined.

In summary, together with the majority of other studies, the present results indicate that the septal nucleus plays an important role in the regulation of experimental fear or anxiety. Furthermore, these results suggest that septal NMDA and non-NMDA receptors are differentially involved in the regulation of specific fear reactions. In particular, it appears that non-NMDA, but not NMDA, receptors regulate rats' fear reactions in the elevated plus-maze test. On the other hand, both non-NMDA and NMDA receptors might be involved in regulating the burying response. Finally, further

investigation into the anomalous effect of AP-5 on rats' shock-probe avoidance may shed light on how activity in different regions of the brain is integrated into adaptive responding. Accordingly, the possibility that intra-septal AP-5 indirectly increased shock-probe contacts by disinhibiting GABAergic projections from the septum to the amygdala merits further research.

Figure 3-1. Histological results of rats infused with EAA antagonists into the septal nucleus. Circles indicate the location of the cannulae tips for saline (open circles), AP-5 (filled circles), and CNQX (stippled circles) infusions. Numbers indicate sections anterior to bregma, adapted from Paxinos and Watson, 1986.

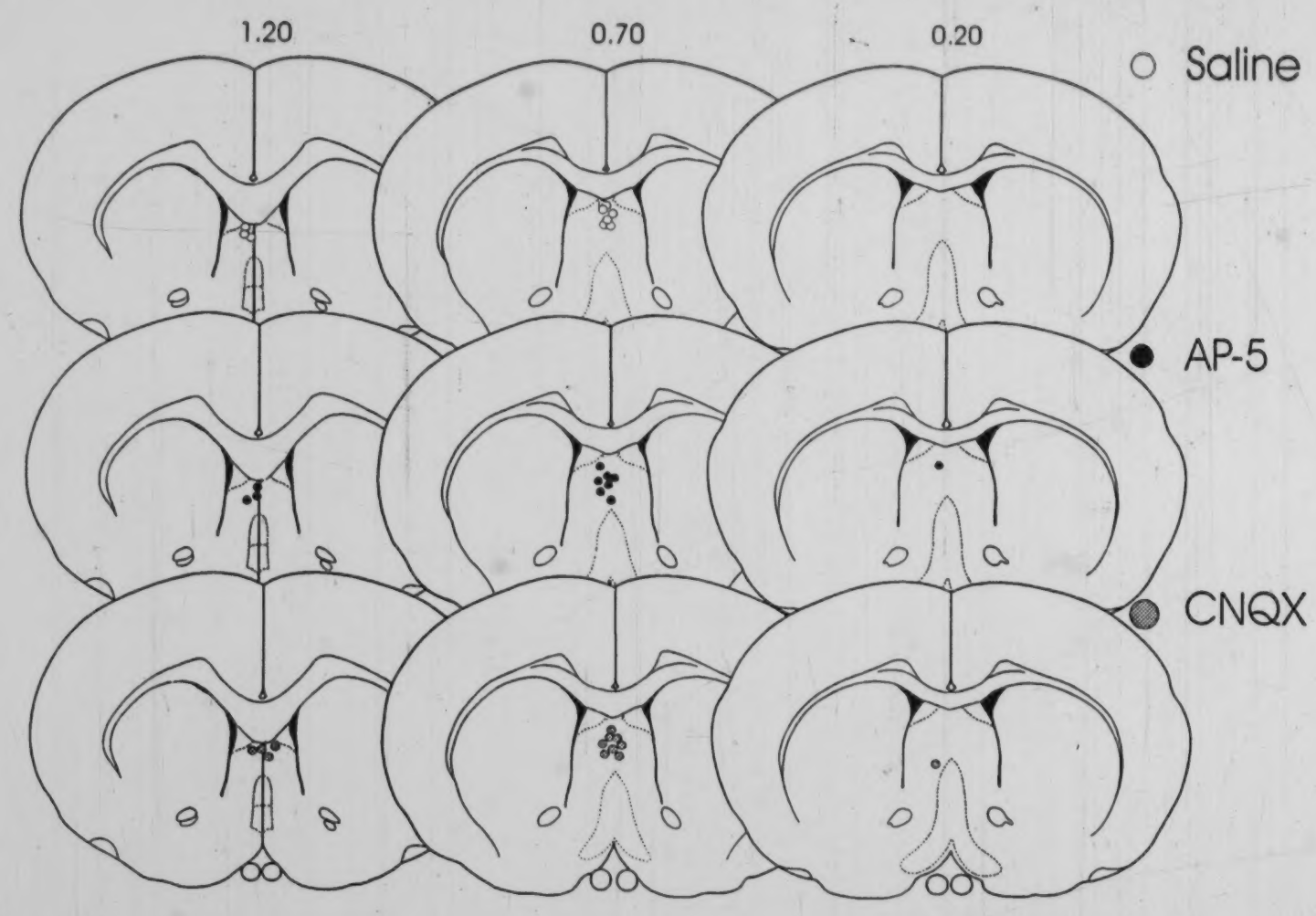

1.20
0.70
0.20
Saline
AP-5
CNQX

Figure 3-2. Mean (± s.e.m.) percentage of open-arm entries (open bars) and percentage of open-arm time (stippled bars) in the plus-maze after intra-septal infusions of AP-5 or CNQX (*$p < 0.05$ compared with controls).

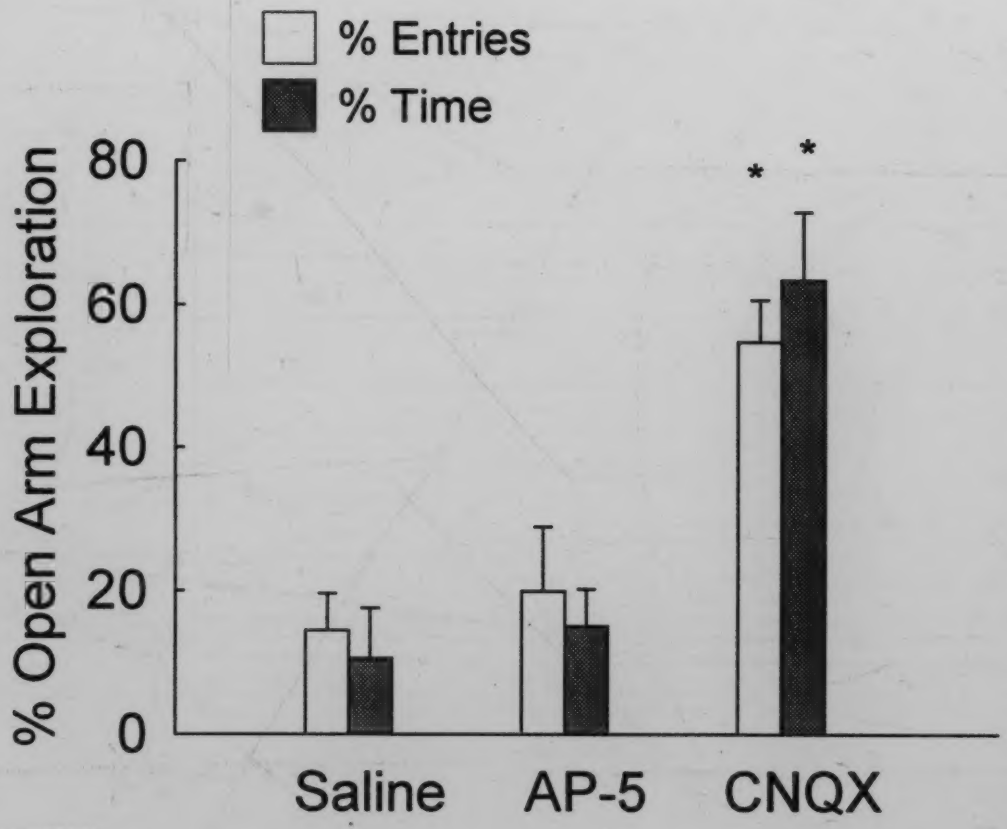

% Entries
% Time
% Open Arm Exploration
80
60
40
20
0
*
*
Saline
AP-5
CNQX

Table 3 - 1

Mean (± s.e.m.) Activity and reactivity scores after intra-speptal infusions of Saline, CNQX (5 μg), or AP-5 (5 μg); *p <0.05 compared to control.

	Treatment		
Behavior	Saline	CNQX	AP-5
Closed-Arm Entries	7.9 (± 0.94)	5.4 (± 1.61)	6.9 (± 1.39)
Shock-reactivity	2.17 (± 0.21)	2.04 (± 0.23)	2.86 (± 0.12)*
Immobility	26.9 (± 12.9)	146.4 (± 51.8)*	51.1 (± 26.46)

Figure 3-3. Mean (± s.e.m.) duration of burying (log s) after intra-septal infusions of AP-5 or CNQX (*$p < 0.05$ compared with controls).

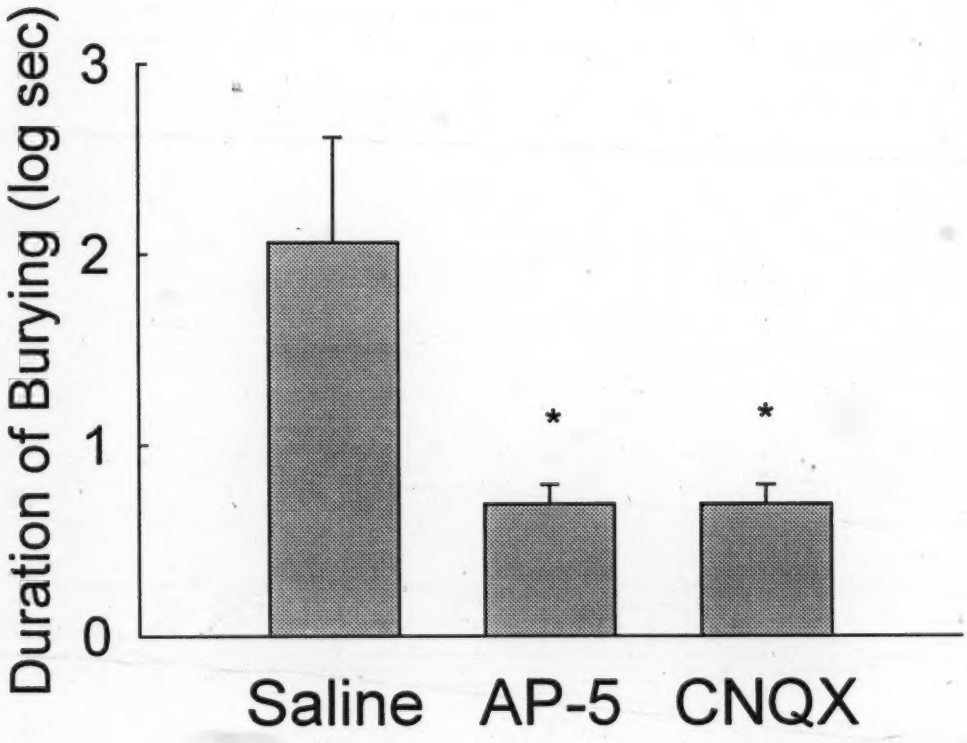

Duration of Burying (log sec)
3
2
1
0
*
*
Saline
AP-5
CNQX

Figure 3-4. Mean (± s.e.m.) number of shocks received by rats after intra-septal infusions of AP-5 or CNQX (*$p < 0.05$ compared with controls).

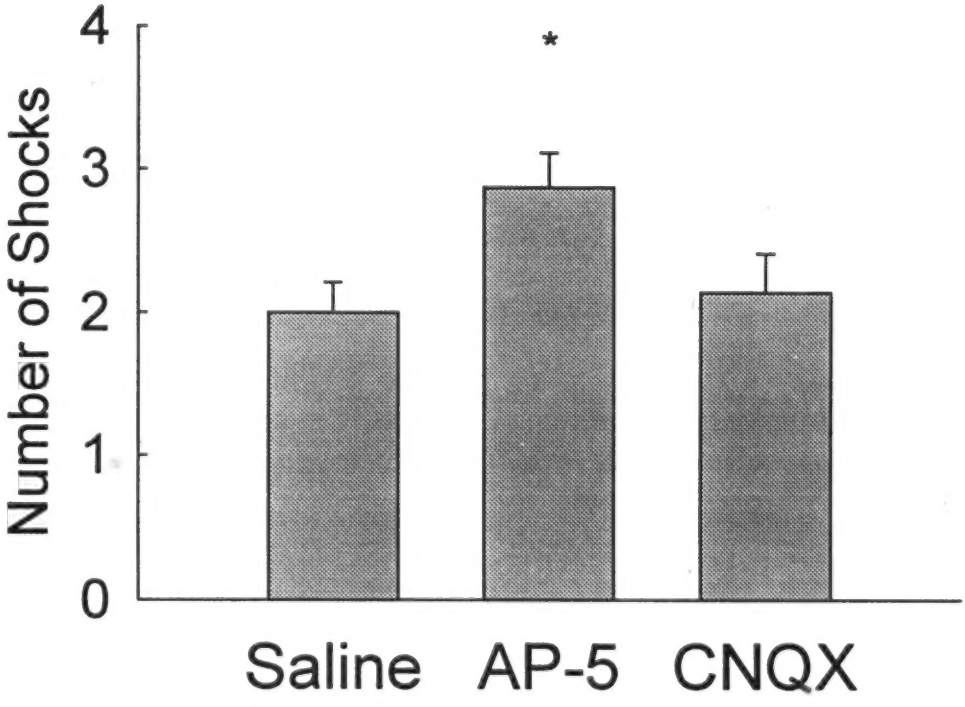

Number of Shocks
4
3
2
1
0
*
Saline
AP-5
CNQX

Figure 3-5. Histological results of rats infused with AP-5 into the septal nucleus. Circles indicate the location of the cannulae tips for saline (open circles) and AP-5 (filled circles), infusions. Numbers indicate sections anterior to bregma, adapted from Paxinos and Watson, 1986.

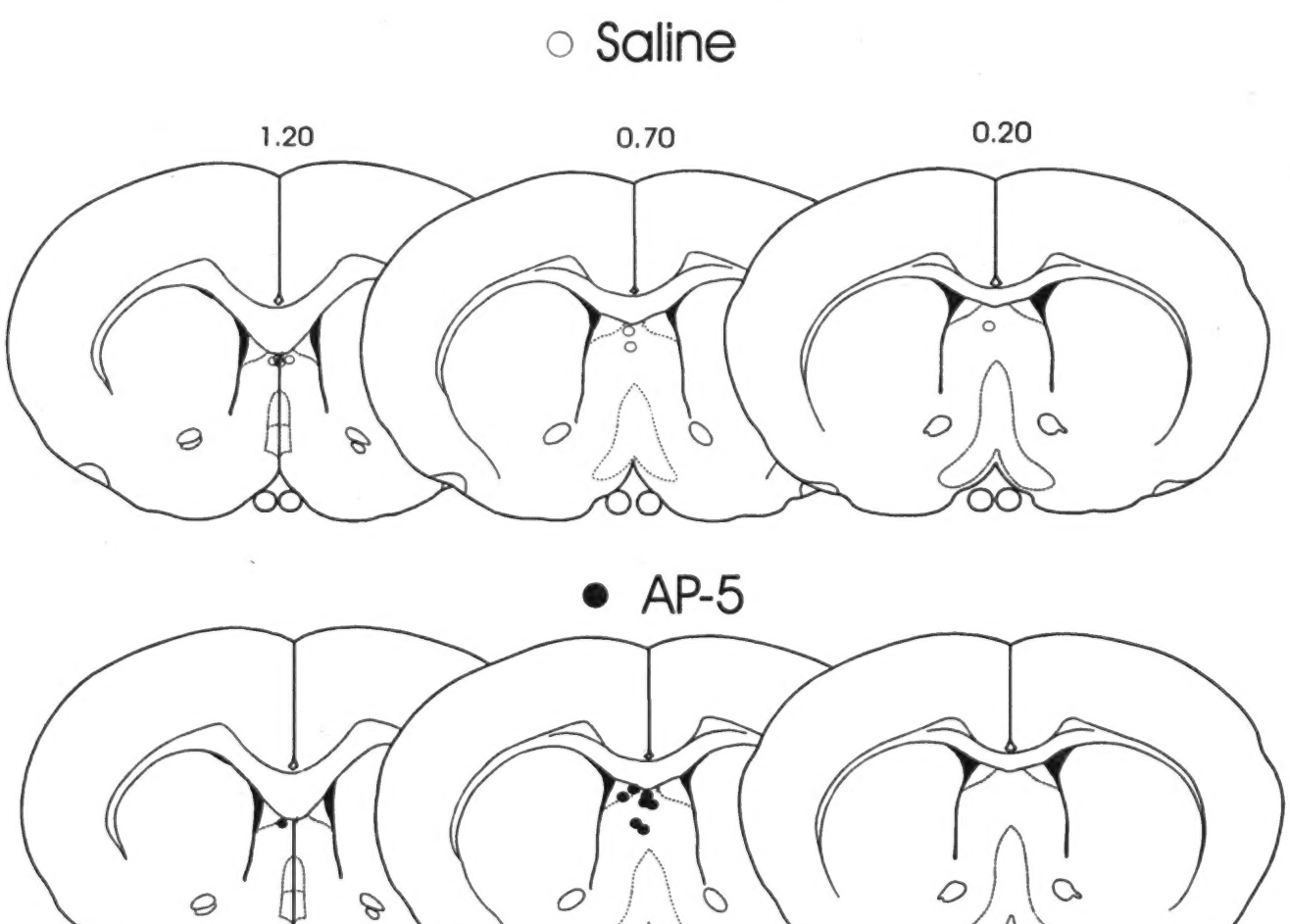
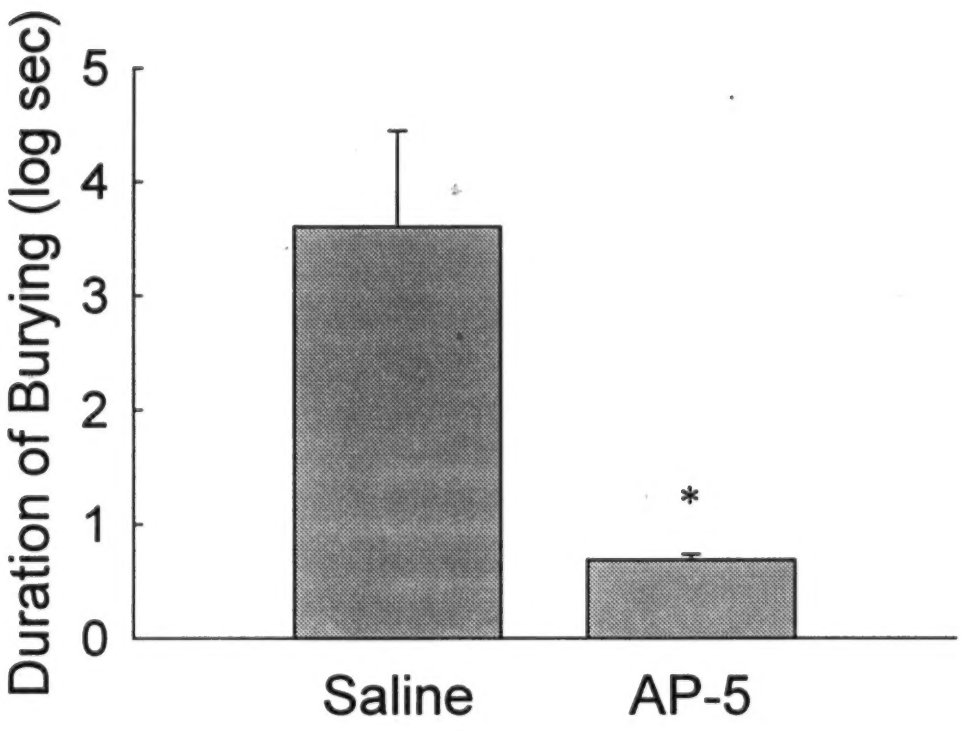

○ Saline
1.20
0.70
0.20
● AP-5

Figure 3-6. Mean (± s.e.m.) duration of burying (log s; top panel) and number of shocks received (bottom panel) after intra-septal infusions of AP-5 (*$p < 0.05$ compared with controls).


Duration of Burying (log sec)
5
4
3
2
1
0
*
Saline
AP-5

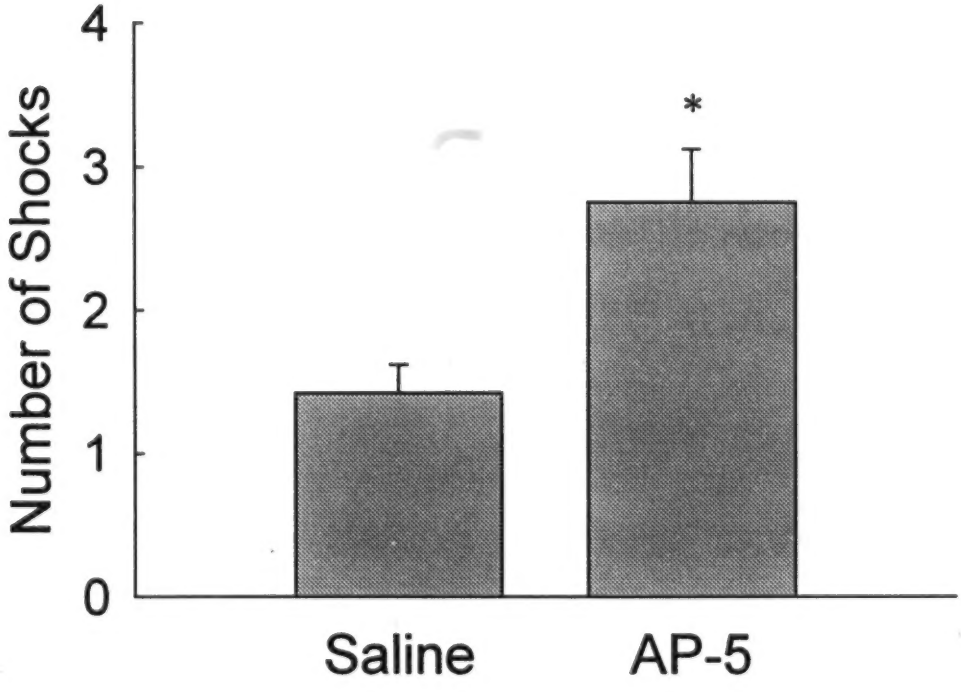

Number of Shocks
4
3
2
1
0
*
Saline
AP-5

Table 3 - 2

Mean (± s.e.m.) Activity and reactivity scores after intra-septal infusion of saline or AP-5 (5 µg).

	Treatment	
Behavior	Saline	AP-5
Shock-reactivity	2.57 (± 0.28)	2.68 (± 0.29)
Immobility	20.7 (± 11.0)	12.7 (± 3.6)

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Chapter 4

The anxiolytic effects of intra-hippocampal midazolam

Introduction

Since the discovery of brain receptors for benzodiazepine-type anxiolytics in 1977 (Mohler & Okada, 1977; Squires & Braestrup, 1977), there has been a growing interest in localizing the anxiolytic properties of these compounds to particular parts of the brain. Classic benzodiazepines (e.g., midazolam) act as full agonists at the benzodiazepine (BZ) receptor, which exists as a unique binding site on the GABAA receptor complex (Sieghart, 1992). Agonist stimulation of the BZ receptor site ultimately results in a facilitation of the inhibitory actions of GABA (e.g., Smith & Olsen, 1995; Zorumsky & Isenburg, 1991). Although widely distributed throughout the central nervous system, BZ receptors are particularily abundant in limbic structures; e.g., septum, hippocampus and amygdala (Neihoff & Kuhar, 1983; Young & Kuhar, 1980). Because these structures have long been implicated in the control of anxiety (e.g., Davis, 1992; Gray, 1982, 1991; Kluver & Bucy, 1937; LeDoux, 1996), it comes as no surprise that they have been regarded as candidate loci for mediating the anxiolytic effects of benzodiazepines. Indeed, several studies have shown that direct application of benzodiazepine-type anxiolytics into specific regions of the limbic system can suppress rats' fear reactions in a variety of animal models of anxiety (for a review, see Menard & Treit, in press).

However, a disproportionate number of these studies have focused on the role of BZ receptors in amygdala in the regulation of fear or anxiety. For example, intra-amygdala infusions of various benzodiazepine agonists (e.g., diazepam, midazolam, chlordiazepoxide, flurazepam and lormetazepam) have been shown to suppress fear behaviors in the social interaction, light-dark exploration, elevated plus-maze, open field,

and Geller or Vogel conflict tests of animal 'anxiety' (Costall et al., 1989; Higgins et al., 1991; Hodges, Green, & Glenn, 1987; Gonzalez, Andrews, & File, 1996; Green & Vale, 1992; McNamara & Skelton, 1993; Mesches, Bianchin, & McGaugh, 1996; Nagy, Zambo, & Desci, 1979; Pesold & Treit, 1995; Peterson, Braestrup, & Sheel-Kruger, 1985; Scheel-Kruger & Peterson, 1982; Shibata et al., 1982, 1989; Thomas, Lewis, & Iversen, 1985; Zangrossi Jr. & Graeff, 1994). In comparison, far less is known regarding the contribution of other limbic structures, such as the septum and hippocampus, to benzodiazepine-induced anxiolysis (Menard & Treit, in press). The absence of such data may be critical, because recent evidence suggests that BZ receptors in some areas of the brain (e.g., septum) regulate different fear reactions than BZ receptors located in other areas (e.g., amygdala). For example, although midazolam infusions into the lateral septum increased rats' open-arm exploration in the elevated plus-maze and completely suppressed their burying response in the shock-probe burying test, infusions of midazolam into the central amygdala produced neither of these anxiolytic effects (Pesold & Treit, 1994, 1995, 1996). Central amygdala infusions of midazolam did, however, seriously impair rats' avoidance of the electrified probe in the burying test, an anxiolytic effect not produced by intra-septal midazolam (Pesold & Treit, 1994, 1995). Furthermore, infusions of midazolam into the basolateral amygdala increased open-arm exploration but failed to impair either shock-probe avoidance or burying behavior (Pesold & Treit, 1995). Although complex, these dissociations suggest that BZ receptors in different limbic structures exert distinct control over different fear behaviors.

The present study was designed to extend these findings by examining the effects

of midazolam infusions into the hippocampus on rats' fear responses in the elevated plus-maze and shock-probe burying tests. Anatomically, the hippocampus is intimately connected with the septum (e.g., Nauta & Domesick, 1982; Risold & Swanson, 1997a; Swanson & Cowan, 1979), and together they form a large part of the limbic system. Functionally, according to Gray's theory (Gray, 1982, 1991), the hippocampus and septum share common control over anxiety, as evidenced in part by the remarkable correspondence between the effects of septal or hippocampal lesions in traditional aversive learning paradigms and the effects of anxiolytic drugs in the same paradigms (for reviews, see Gray, 1982; Gray & McNaughton, 1983). In fact, Gray's theory would predict that benzodiazepine receptors in the septum and the hippocampus should play similar roles in regulating fear responses. If this is the case, then intra-hippocampal infusions of midazolam should produce a pattern of results similar to that produced by intra-septal midazolam; i.e., increases in open-arm exploration and decreases in burying behavior. If, however, these structures differentially mediate the anxiolytic actions of benzodiazepines, then dissociations in this pattern of results might emerge.

EXPERIMENT 1

Methods

Subjects

Subjects were naive, Sprague Dawley rats (Charles River, Canada) weighing between 250-300 g at the time of surgery. Following surgery, the rats were returned to

the animal colony where they were maintained in the same manner as outlined in Chapter 2.

Surgery and histology

Anethetized rats (n = 32) were bilaterally implanted with guide cannulae aimed 1 mm above the dorsal hippocampus (3.1 mm posterior and ± 2.6 mm lateral to bregma, 3 mm ventral to dura, with the guide cannulae angled 20° towards the midline; flat skull coordinates from Paxinos & Watson, 1986). All other surgical and histological methods were the same as in Chapter 2.

Behavioral testing

Behavioral testing was the same as in Chapter 2, with the following exceptions. Following 10 days of post-surgical recovery, handling-habituated rats received their first infusions, 5 min prior to being tested in the elevated plus-maze. Seven days later, rats received their second infusion, 5 min prior to being tested in the shock-probe burying test.

Infusion procedures and drug regimens

Infusion procedures were the same as in Chapter 2. Midazolam maleate (donated by Roche Products) was dissolved in 0.9 % saline. The drug dose (10 µg/side) was chosen on the basis of previous work in this laboratory (Pesold & Treit, 1994). Rats were infused with 1 µl of solution/side, at an infusion rate of 1µl/min, with the infusion cannula remaining in place for an additional 1 min diffusion period. Rats were randomly assigned

to their respective treatment groups and received the same treatment prior to each test. Some rats were not tested in both paradigms due to post-infusion fluid efflux from the end of a cannula guide or failure to contact the electrified probe in the burying test. The number of animals in each treatment group depicted below had verified cannulae placements (Figure 4-1).

Before the plus-maze test, rats received intra-hippocampal infusions of either 1 µl/side of 0.9 % saline (n = 14) or 10 µg/side of midazolam (n = 13). Before the shock-probe burying test, the same rats received 1 µl/side of 0.9% saline (n = 14) or 10 µg/side of midazolam (n = 8).

Statistical analysis

Statistical analysis was the same as in Chapter 2. Specifically, results were analyzed using one-way analsysis of variance (ANOVA). In order to correct for non-normality and heterogenity of variance, the duration of burying scores were transformed (natural log) prior to the ANOVA.

Results

Elevated plus-maze. As can be seen in Figure 4-2, intra-hippocampal infusions of midazolam (10 µg/side) produced substantial, significant increases in both the percentage of entries rats made into the open-arms and the percentage of time rats spent on the open arms; ANOVA [**percent open-arm entries**: $F(1,25) = 6.83$, $p < 0.05$; **percent open-arm time**; $F(1,25) = 12.39$, $p < 0.01$]. These midazolam-induced increases in open-arm

exploration appeared specific to fear reduction because there were no concomitant changes general activity; ANOVA [**closed-arm entries**; $F(1,25) = 0.10$, $p > 0.74$; see Table 4-1].

Shock-probe burying test. In contrast to its clear anxiolytic effects in the elevated plus-maze, bilateral infusion of midazolam (10 μg/side) into the dorsal hippocampus failed to alter any behaviors measured in the shock-probe burying test; ANOVA [**burying**; $F(1,20) = 0.13$, $p > 0.71$; **number of shocks**; $F(1,20) = 0.12$, $p > 0.72$; **immobility**; $F(1,20) = 1.56$, $p > 0.22$; **shock reactivity**; $F(1,20) = 0.04$, $p > 0.81$; see Table 4-1]. Overall, these results suggest that BZ receptors in the dorsal hippocampus are involved in the regulation of rats' fear reactions in the plus-maze but not in the shock-probe burying test.

EXPERIMENT 2

Combined with previous findings showing that direct application of midazolam into the lateral septum increases open-arm exploration and decreases burying behavior (Pesold & Treit, 1994, 1996), the current results suggest that the dorsal hippocampus and the lateral septum share common control over rats' fear responses in the elevated plus-maze, but not their fear responses in the shock-probe burying test. However, it is not clear from these results whether septo-hippocampal control of anxiety responses is exerted directly; i.e., via direct connections from the dorsal hippocampus to the lateral septum (Risold & Swanson, 1997a) or indirectly, i.e., via the separate influence of each of these structures at a common projection site (e.g., supramammillary nucleus; Risold & Swanson,

1996). Thus, the purpose of Experiment 2 was to further examine the integrated role of the septum and dorsal hippocampus in regulating rats' fear behaviors in the elevated plus-maze. In this regard, it is interesting to note that the lateral septum receives substantial excitatory amino acid (EAA) input from the hippocampus that is believed to be glutamatergic (Walaas & Fonnum, 1980; Joels & Urban, 1984; Stevens & Cotman 1986). Furthermore, blocking EAA neurotransmission in the septal nucleus, by local infusion of the non-NMDA receptor antagonist, CNQX has been shown to selectively reduce rats' fear behaviors in the elevated plus-maze (Menard & Treit, 1998a; Chapter 3). Thus, it could be hypothesized that the anxiolytic effects of intra-hippocampal midazolam on open-arm avoidance were due to its inhibition of hippocampal glutamatergic efferents to the septal nucleus. If this is the case, then the anxiolytic actions of intra-hippocampal midazolam should be reversed by intra-septal infusions of glutamate.

Methods

Methods and materials were the same as in the preceding experiment (Experiment 1; Chapter 4), with the following exceptions. Twenty, anesthetized, naive Sprague Dawley rats (Charles River, Canada), weighing between 285-310 g, were surgically implanted with a single guide cannula aimed 1 mm above the septal nucleus (0.7 mm anterior and 0.4 mm lateral to bregma, 3 mm ventral to dura with the cannula angled 4° towards the midline). An additional 48 rats were surgically implanted with 3 guide cannulae, 1 of which was aimed 1 mm above the septal nucleus using the coordinates given above and 2 of which were aimed, bilaterally, 1 mm above the dorsal hippocampus

(3.6 mm posterior and ± 2.6 mm lateral to bregma, 2.2 mm ventral to dura with the guide cannulae angled 20° towards the midline). Midazolam maleate (donated by Roche Products) and L-glutamate (RBI) were disolved in 0.9% saline. All solutions were infused at a volume of 1 µl/cannula guide, at a rate of 1µl/min, followed by an additional 1 min diffusion period. After 13 days post-surgical recovery, rats received their appropriate infusions (detailed below), followed 3 min later by a 5 min trial in the elevated plus-maze. One animal with a single (i.e., septal) implant was not tested due to a lost head cap. Eleven animals with triple implants were not tested due to an occluded cannula guide (n = 1), fluid efflux from the end of a cannula guide (n = 5), a split in the polyethylene tubing (n = 1) and excessive handling reactivity (n = 4). Rats were randomly assigned to the treatment groups depicted below (all n's represent accurate guide placements; see Figures 4-3, 4-4, and 4-5). Just prior to the plus-maze test, rats were given an infusion of either 0.9% saline (n = 9) or 3.5 µg of L-glutamate (n = 9) into the septal nucleus or bilateral infusions of midazolam (10 µg/side) into the dorsal hippocampus immediately followed by an infusion of either 0.9% saline (MDZ/SAL; n = 22) or 3.5 µg of L-glutamate (MDZ/GLU; n = 12) into the septal nucleus.

Results

Elevated plus-maze. Rats given intra-hippocampal infusions of midazolam (10 µg/side) followed immediately by intra-septal infusions of 3.5 µg of L-glutamate (MDZ/GLU) showed substantially less open-arm activity in the elevated plus-maze than did rats given hippocampal infusions of midazolam followed by septal infusions of saline (MDZ/SAL;

Figure 4-6). Between groups ANOVA confirmed that both the percentage of entries made into the open arms and the percentage of time spent on the open-arm entries were significantly reduced in MDZ/GLU rats, relative to MDZ/SAL rats [**percent open-arm entries**: $F(1,31) = 7.69$, $p < 0.01$; **percent open-arm time**; $F(1,31) = 7.18$, $p < 0.05$]. The effects of glutamate did not appear secondary to a motor impairment because general activity levels of MDZ/GLU [mean 8.8 (s.e.m. ± 1.41)] and MDZ/SAL [mean 7.5 (s.e.m. ± 0.67)] rats did not differ; ANOVA [**closed-arm entries**: $F(1,31) = 0.89$, $p > 0.35$]. Furthermore, when given alone, the same dose of intra-septal glutamate had no effects on rats' behavior in the elevated plus-maze; i.e., rats given intra-septal infusions of saline or glutamate (3.5 μg) did not differ on any measures taken in this test; ANOVA [**percent open-arm entries**: $F(1,16) = 0.52$, $p > 0.5$; **percent open-arm time**; $F(1,16) = 1.45$, $p > 0.24$; **closed-arm entries**; $F(1,16) = 1.35$, $p > 0.26$; see Table 4-2]. These latter results suggest that the glutamate-induced reductions in open-arm activity displayed by rats given midazolam in the hippocampus were not secondary to glutamate-induced increases in fear (i.e.,anxiogenisis). Overall, these results suggest that hippocampal infusions of midazolam increase open-arm exploration by inhibiting hippocampal glutamatergic efferents to the septal nucleus.

In order to further characterize the effects of midazolam in the dorsal hippocampus, rats from the MDZ/SAL group were sorted into two groups on the basis of their cannulae placements; i.e., those that had both cannulae situated at or anterior to AP = -3.6 (anterior group; n = 11) and those that had one or both cannulae situated posterior to AP = - 3.6 (posterior group; n = 10). Because rats in the previous intra-hippocampal

midazolam study (Experiment 1; Chapter 4) had predominantly anterior placements (as defined above), their measures of open-arm activity are depicted along side those of the anterior and posterior MDZ/SAL groups for comparative purposes (see Figures 4-7 and 4-8). As clearly shown in Figures 4-7 and 4-8, rats infused with midazolam into the anterior dorsal hippocampus (Experiments 1 & 2) displayed substantially more open-arm activity than did rats infused with midazolam into the posterior hippocampus (Experiment 2). These impressions were confirmed by statistical analysis comparing open-arm activity of the anterior and posterior groups from Experiment 2. Specifically, midazolam-treated rats in the anterior group made a significantly greater percentage of entries onto the open-arms and tended to spend a greater percentage of time on the open arms than did midazolam-treated rats in the posterior group; ANOVA [**percent open-arm entries**: $F(1,19) = 5.43$, $p < 0.05$; **percent open-arm time**; $F(1,19) = 3.88$, $p < 0.06$], whereas general activity levels between these groups did not differ; ANOVA [**closed-arm entries**; $F(1,19) = 1.22$, $p > 0.28$]. Finally, it is important to note that the effects of intra-septal glutamate in midazolam-treated rats were not confounded by cannulae placements in the hippocampus. Specifically, substantial, selective reductions in open-arm activity were evident in MDZ/GLU rats with anterior placements ($n = 8$) relative to MDZ/SAL rats with anterior placements ($n = 11$); ANOVA [**percent open-arm entries**: $F(1,17) = 14.27$, $p < 0.01$; **percent open-arm time**; $F(1,17) = 12.03$, $p < 0.01$; **closed arm entries**: $F(1,17) = 2.80$, $p > 0.10$; data not shown].

Discussion

Direct application of midazolam into the dorsal hippocampus selectively increased rats' open-arm exploration in the elevated plus-maze, whereas this same treatment did not alter rats' burying behavior in the shock-probe burying test (Experiment 1). Conversely, intra-septal infusions of glutamate (although without effects when given alone) selectively suppressed the open-arm exploration of rats given midazolam infusions into the dorsal hippocampus (Experiment 2). In combination, these results suggest that the anxiolytic effects of intra-hippocampal midazolam in the plus-maze might be due to a midazolam-induced inhibition of glutamatergic efferents from the dorsal hippocampus to the lateral septum.

The midazolam-induced increases in open-arm exploration observed in Experiment 1 appeared specific to fear reduction, given the absence of drug effects on general activity levels (i.e., closed-arm entries did not differ between groups). Furthermore, when midazolam- and midazolam/saline-treated rats (from Experiments 1 and 2, respectively) were equated for cannulae placements, they showed remarkably similar degrees of open-arm exploration (see Figures 4-7 and 4-8), which might suggest that intra-hippocampal midazolam was similarly anxiolytic in Experiment 2. At any rate, the present results agree with previous demonstrations of anxiolysis, in the open field and Vogel conflict tests, following local application of benzodiazepine agonists into the dorsal hippocampus (Kataoka et al., 1991; Plaznik et al., 1994; Stefanski et al., 1993).

It is not readily apparent why midazolam/saline-treated rats with posterior cannulae placements explored the open arms less than similarly treated rats with anterior

cannulae placements (Experiment 2). On the one hand, projections from hippocampus to lateral septum are known to be topographically organized along a dorsoventral axis, i.e., progressively more ventral parts of the hippocampus innervate progressively more ventral parts of the lateral septum (e.g., Risold & Swanson, 1997a). Perhaps, midazolam infusions in the posterior hippocampus diffused in a more ventral direction than did anterior infusions, thus inhibiting hippocampal areas which might not be associated with rats' open-arm avoidance. Alternatively, projections from the dorsal half of the hippocampus to the lateral septum originate exclusively in area CA3. Because area CA3 was relatively more lateral to the cannula tips in posterior than in anterior placements, it seems possible that the concentration of midazolam reaching CA3 following posterior infusions was insufficient to induce anxiolysis. Additional work is needed to choose between these alternatives.

In Experiment 2, infusions of glutamate into the septal nucleus clearly reduced the open-arm activity of rats treated with midazolam in the dorsal hippocampus. It is important to note that if intra-septal glutamate was anxiogenic, then this could mask the anxiolytic actions of intra-hippocampal midazolam regardless of whether these latter effects were ultimately due to reduced neurotransmission in a hippocampal projection target other than the septal nucleus (e.g., supramammillary nucleus). Furthermore, although glutamate-induced anxiogenisis was not apparent in this study, (i.e., rats given saline or glutamate into the septum explored the open arms to a similar degree), baseline levels of open-arm activity shown by saline treated rats were relatively low (< 10%), which could have precluded the detection of anxiogenic effects. Nevertheless, the

interpretation that intra-hippocampal midazolam is anxiolytic because it reduces glutamate release in the septum (or, conversely, that intra-septal glutamate reverses the anxiolytic actions of hippocampal midazolam by replenishing glutamate levels in the septum) is consonant with the known connectional and functional characteristics of the septal nucleus. First, the major source of inputs to the lateral septum has been shown to originate in the hippocampus and to be excitatory (most likely glutamatergic) in nature (for reviews see Gallagher et al., 1995; Risold & Swanson, 1997a,b). Second, a uniformly 'excitatory' role has been established for the septum in the regulation of rats' fear behaviors in the elevated plus-maze (for reviews see Chapter 1; Treit & Menard, in press). Finally, intra-septal infusions of the glutamate receptor antagonist, CNQX have been shown to produce clear, anxiolytic effects in the plus-maze test (Chapter 3). Given this overall view, the current results suggest that the hippocampus regulates rats' fear responses in the plus-maze via a direct hippocampal influence (i.e., glutamate release) on the septal nucleus. However, it remains possible that other hippocampal targets may be similarly involved in the anxiolytic actions of intra-hippocampal midazolam.

Despite a convergent body of evidence that intra-hippocampal midazolam can produce anxiolysis in different animal models of anxiety (see above; Kataoka et al., 1991; Plaznik et al., 1994; Stefanski et al., 1993), including the elevated plus-maze (current results), rats' normal fear responses in the shock-probe burying test were not impaired by intra-hippocampal midazolam (Experiment 1). Interestingly, a similar dissociation was previously observed in this laboratory following dorsal hippocampal infusions of the inhibitory compound, R(+)-8-OH-DPAT, a 5-HT_{1A} receptor agonist; i.e., intra-

hippocampal R(+)-8-OH-DPAT increased rats' open-arm exploration in the plus-maze without impairing their burying response in the shock-probe burying test (Chapter 2; Menard & Treit, 1998b). In light of the extensive evidence implicating the septal nucleus in fear responding in both tests (Chapter 1; Menard & Treit, in press), it appears that the septum and the *dorsal* hippocampus exert common control over some (open-arm avoidance) but not all (burying behavior) fear behaviors. However, it remains entirely possible that other hippocampal regions (e.g., ventral hippocampus) could play a role in the regulation of burying behavior. In this regard, it is interesting to note that area CA3 (in both dorsal and ventral hippocampus) and area CA1, as well as the subiculum (in ventral hippocampus) selectively innervate two distinct regions of the lateral septum; i.e., the newly identified Lateral Septum caudal (LSc) and Lateral Septum rostral (LSr), respectively (Risold & Swanson, 1996; 1997 a,b). Those authors have also shown that the LSc and LSr are distinct not only in terms of their chemoarchitecture, but also in terms of their bidirectional connections with functionally distinct regions of the hypothalamus. Of particular interest, area CA1 and the subiculum, in ventral hippocampus, selectively innervate LSr which, in turn, has selective, reciprocal connections with hypothalamic regions (e.g., the perifornical region; Risold & Swanson, 1996, 1997a; Roeling et al., 1994) previously associated with rats' defensive behaviors towards predators or dominant conspecifics (Roeling et al., 1994). As such, it would be very interesting to determine the role of both the ventral hippocampus and these hypothalamic 'predator defense' zones in burying (and open-arm avoidance). Regardless of the outcome of such studies, it seems likely that the anxiolytic effects of intra-dorsal hippocampal midazolam observed in the

current study were due to disruption of the CA3 to LSc projection, and that this particular projection path regulates open-arm avoidance but not burying behavior.

Finally, in an earlier study it was found that electrolyic lesions of the dorsal hippocampus failed to affect rats' normal open-arm avoidance in the elevated plus-maze (Treit & Menard, 1997; also see Chapter 1). However, the lesions in that study were primarily restricted to area CA1, the dentate gyrus and the subiculum, with substantial sparing of area CA3, which might explain why dorsal-hippocamal lesions (unlike dorsal-hippocampal midazolam) had no effects in the plus-maze test. On the other hand, it is not clear why hippocampal lesions produced a significant increase in shock-probe contacts in the burying test, an anxiolytic effect not observed following intra-dorsal hippocampal midazolam. Additional studies are needed to fully determine the reasons for these apparent discrepencies.

In summary, infusions of the benzodiazepine agonist, midazolam into the dorsal hippocampus had anxiolytic effects in the elevated plus-maze, but not in the shock-probe burying test. This dissociation complements earlier findings observed in this laboratory, i.e., that the lateral septum, central and basolateral amygdala differentially mediate the anxiolytic effects of midazolam (see introduction; Pesold & Treit, 1994, 1995, 1996), and further corroborates the contention that BZ receptors in different limbic regions exert distinct (yet, in some cases, overlapping) control over different fear behaviors. The current results also suggest that rats' open-arm avoidance (but not their burying behavior) is modulated by glutamatergic, septal afferents originating in the dorsal hippocampus. Whether these different fear behaviors are regulated by a common or different septal

efferent projection paths (e.g., to either the same or functionally distinct regions of the hypothalamus) remains to be determined.

Figure 4-1. Histological results of rats infused with midazolam into the dorsal hippocampus. Circles indicate the location of the cannulae tips for saline (open circles), and midazolam (filled circles) infusions. Numbers indicate sections posterior to bregma, adapted from Paxinos and Watson, 1986.

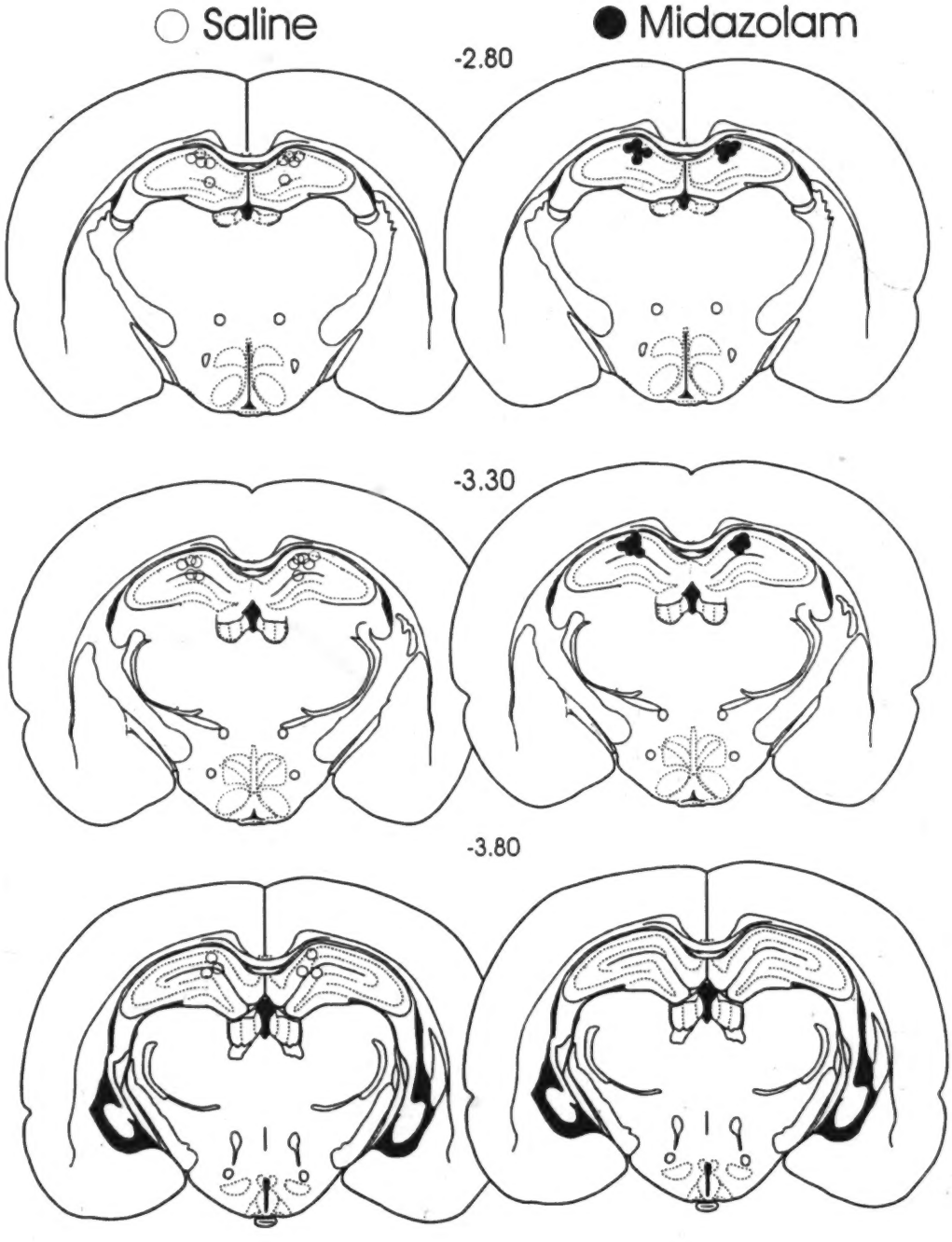

○ Saline
● Midazolam
-2.80
-3.30
-3.80

Figure 4-2. Mean (± s.e.m.) percentage of open-arm entries (open bars) and percentage of open-arm time (stippled bars) in the plus-maze after intra-hippocampal infusions of midazolam (*$p < 0.05$ compared with controls).

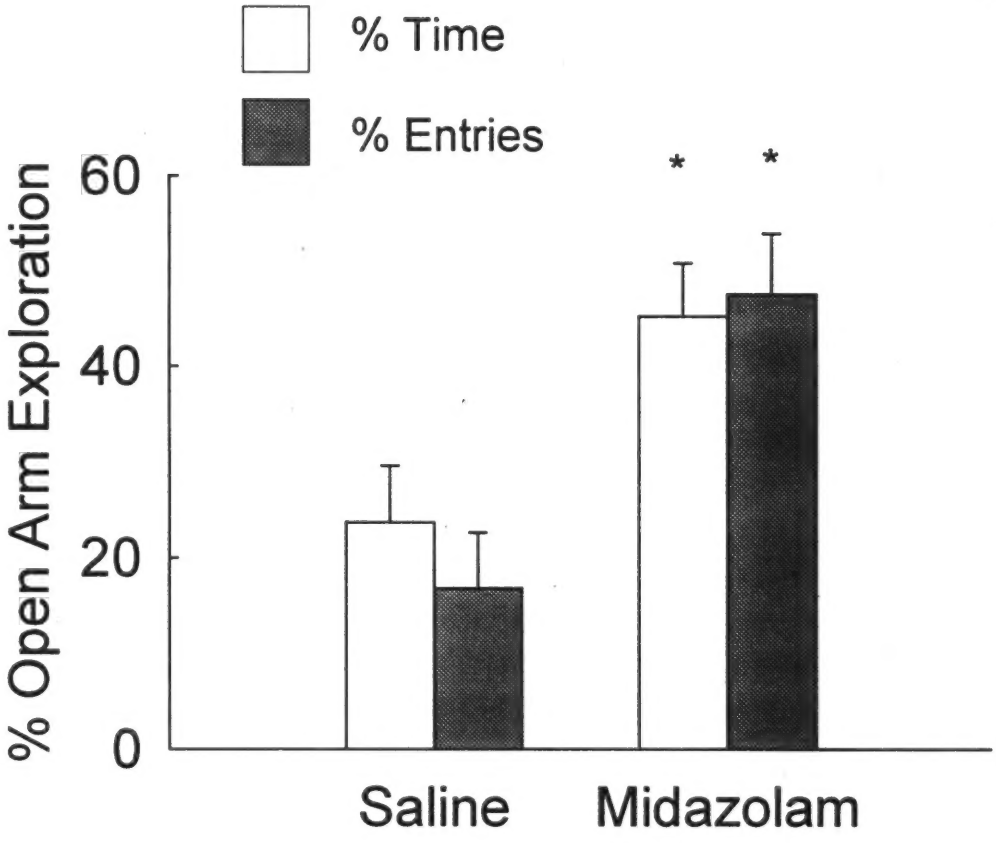

% Time
% Entries
% Open Arm Exploration
60
40
20
0
*
*
Saline
Midazolam

Table 4 - 1

Mean (± s.e.m.) Duration of burying (log sec), activity, and reactivity after intra-hippocampal infusions of saline or midazolam (10 µg/side).

	Treatment	
Behavior	Saline	Midazolam
Duration of Burying	0.83 (± 0.40)	1.09 (± 0.65)
Shock-reactivity	2.13 (± 0.26)	2.04 (± 0.33)
Number of Shocks	1.71 (± 0.30)	1.88 (± 0.29)
Immobility	48.2 (± 13.1)	124.0 (± 78.19)
Closed-Arm Entries	7.2 (± 0.92)	7.6 (± 0.84)

Figure 4-3. Histological results of rats infused with L-Glutamate into the septal nucleus. Circles indicate the location of the cannulae tips for saline (open circles) and L-Glutamate (filled circles) infusions. Numbers indicate sections anterior to bregma, adapted from Paxinos and Watson, 1986.

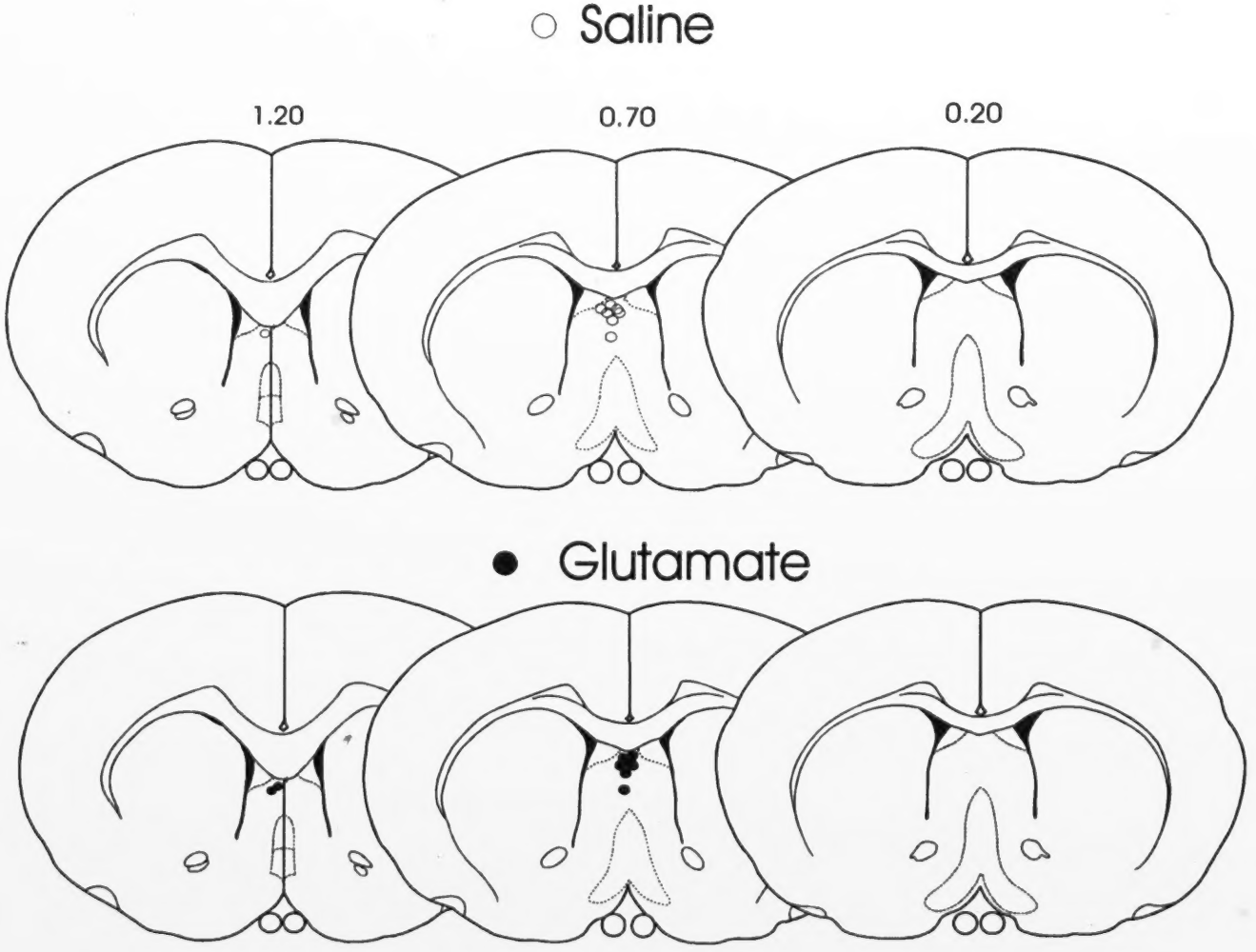

○ Saline
1.20
0.70
0.20
● Glutamate

Figure 4-4. Histological results of rats infused with midazolam into the dorsal hippocampus (top panels) followed by infusions of saline into the septal nucleus (bottom panel). Circles indicate the location of the cannulae tips for midazolam (filled circles) and saline (open circles) infusions. Numbers indicate sections posterior (top panels) and anterior (bottom panel) to bregma, adapted from Paxinos and Watson, 1986.

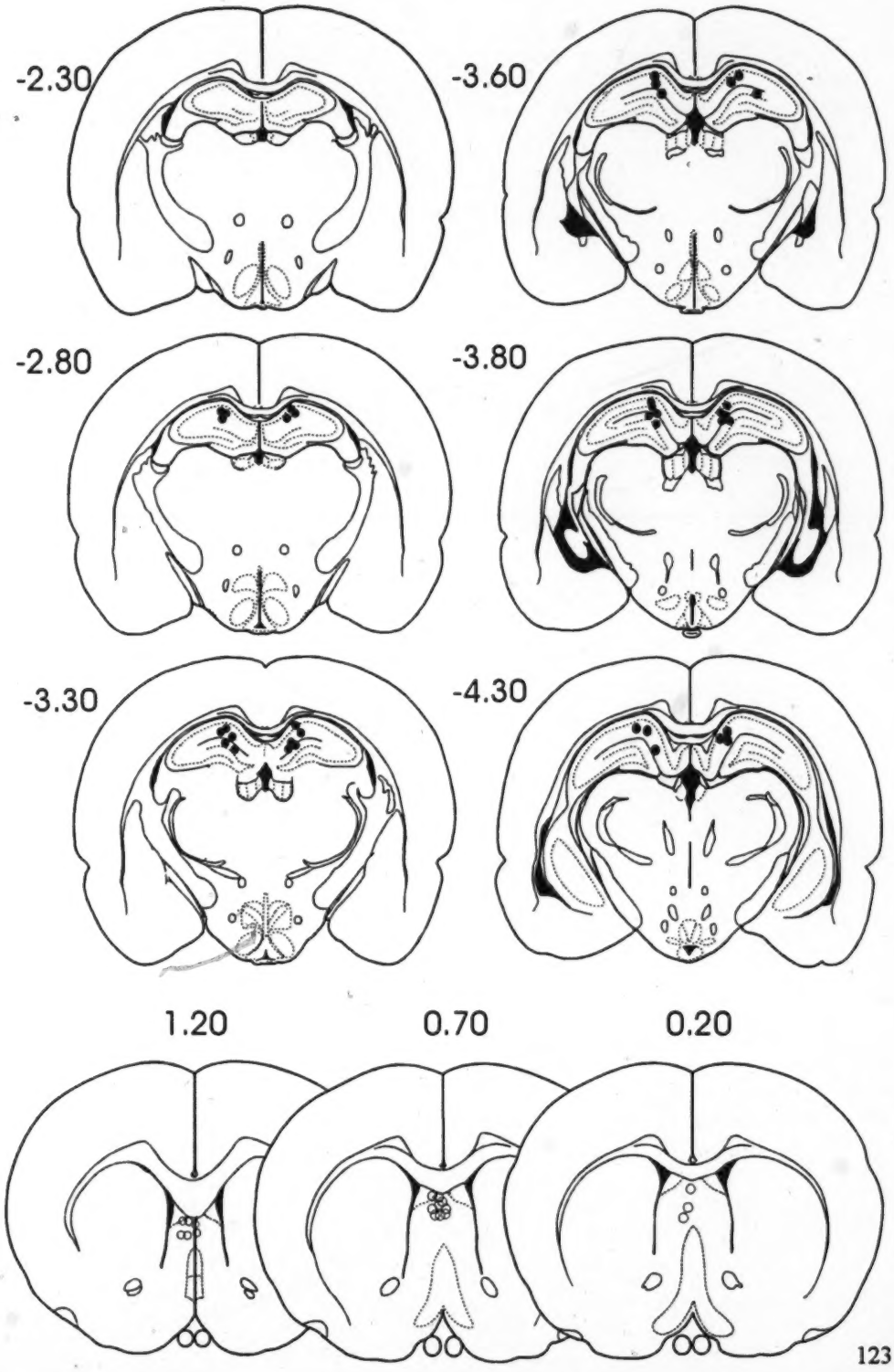

-2.30
-3.60
-2.80
-3.80
-3.30
-4.30
1.20
0.70
0.20

Figure 4-5. Histological results of rats infused with midazolam into the dorsal hippocampus (top panels) followed by infusions of L-Glutamate into the septal nucleus (bottom panel). Circles indicate the location of the cannulae tips for midazolam (filled circles) and L-Glutamate(stippled circles) infusions. Numbers indicate sections posterior (top panels) and anterior (bottom panel) to bregma, adapted from Paxinos and Watson, 1986.

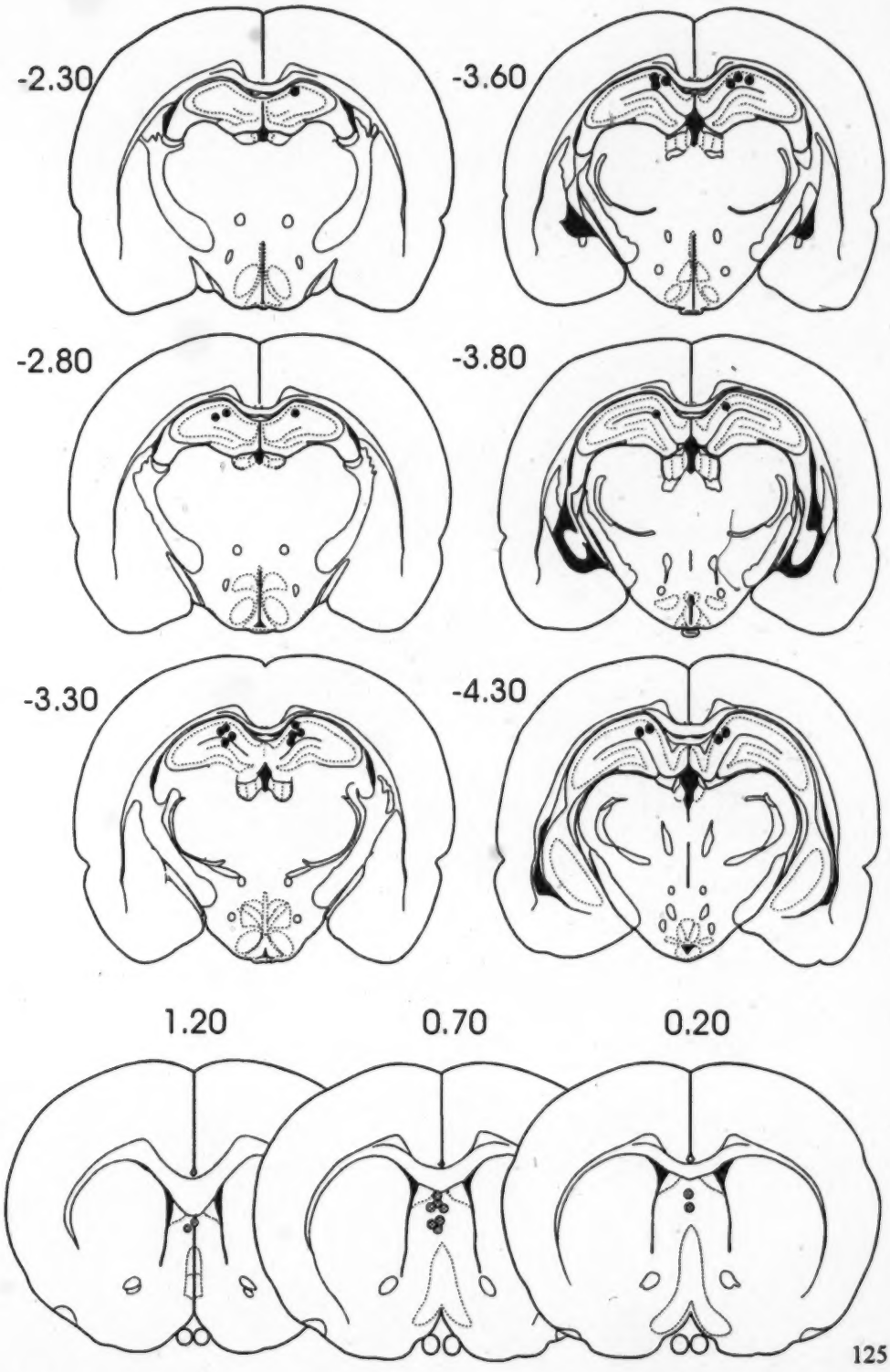

-2.30
-3.60
-2.80
-3.80
-3.30
-4.30
1.20
0.70
0.20

Figure 4-6. Mean (± s.e.m.) percentage of open-arm entries (open bars) and percentage of open-arm time (stippled bars) in the plus-maze after intra-hippocampal infusions of midazolam followed by intra-septal infusions of saline or L-Glutamate (*$p < 0.05$ compared with controls).

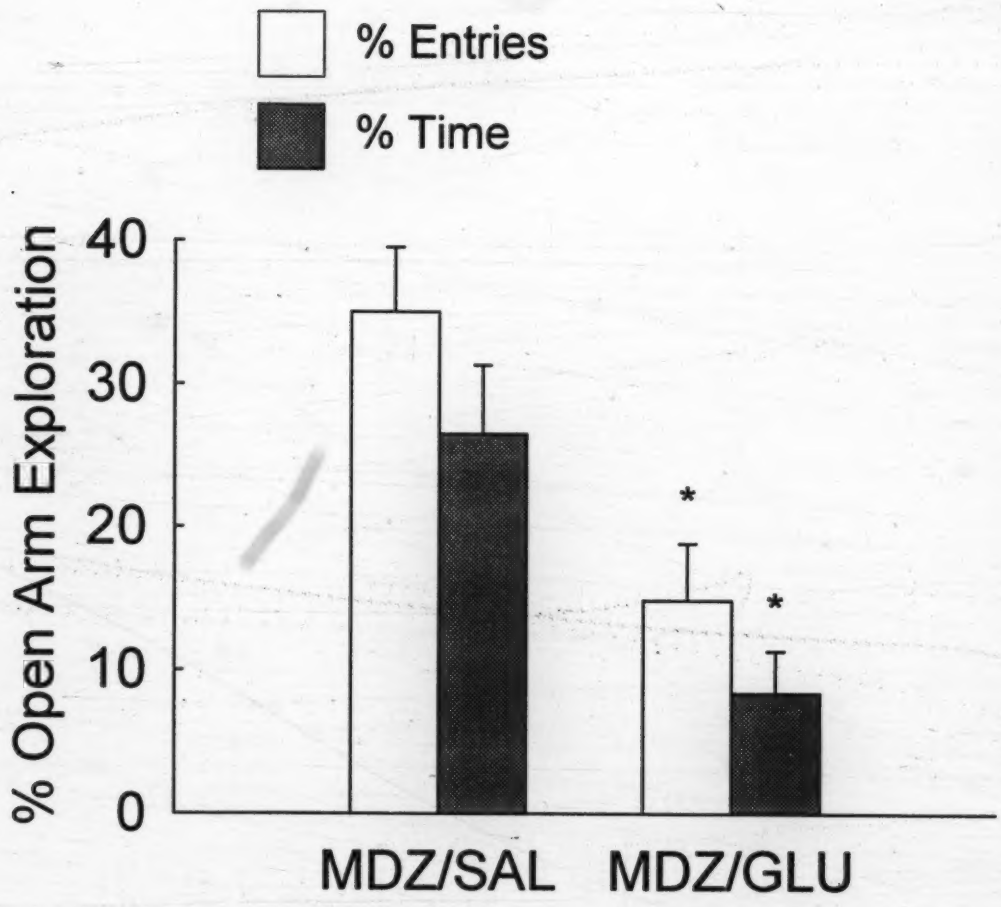

% Entries
% Time
% Open Arm Exploration
40
30
20
10
0
*
*
MDZ/SAL
MDZ/GLU

Table 4 - 2

Mean (± s.e.m) Plus-maze activity after intra-septal infusions of saline or glutamate (3.5 μg).

	Treatment	
Behavior	Saline	Glutamate
% Open-Arm Entries	7.6 (± 3.57)	8.5 (± 2.66)
% Open-Arm Time	5.7 (± 2.44)	2.7 (± 0.83)
Closed-Arm Entries	8.2 (± 0.59)	7.2 (± 0.62)

Figure 4-7. Mean (± s.e.m.) percentage of entries made into the open arms of the plus-maze after infusions of saline (open bars) or midazolam (stippled bars) into the anterior aspects of the dorsal hippocampus (Experiment 1), and infusions of midazolam into the posterior (diagonal bar) or anterior (hatched bar) aspects of the dorsal hippocampus (Experiment 2).

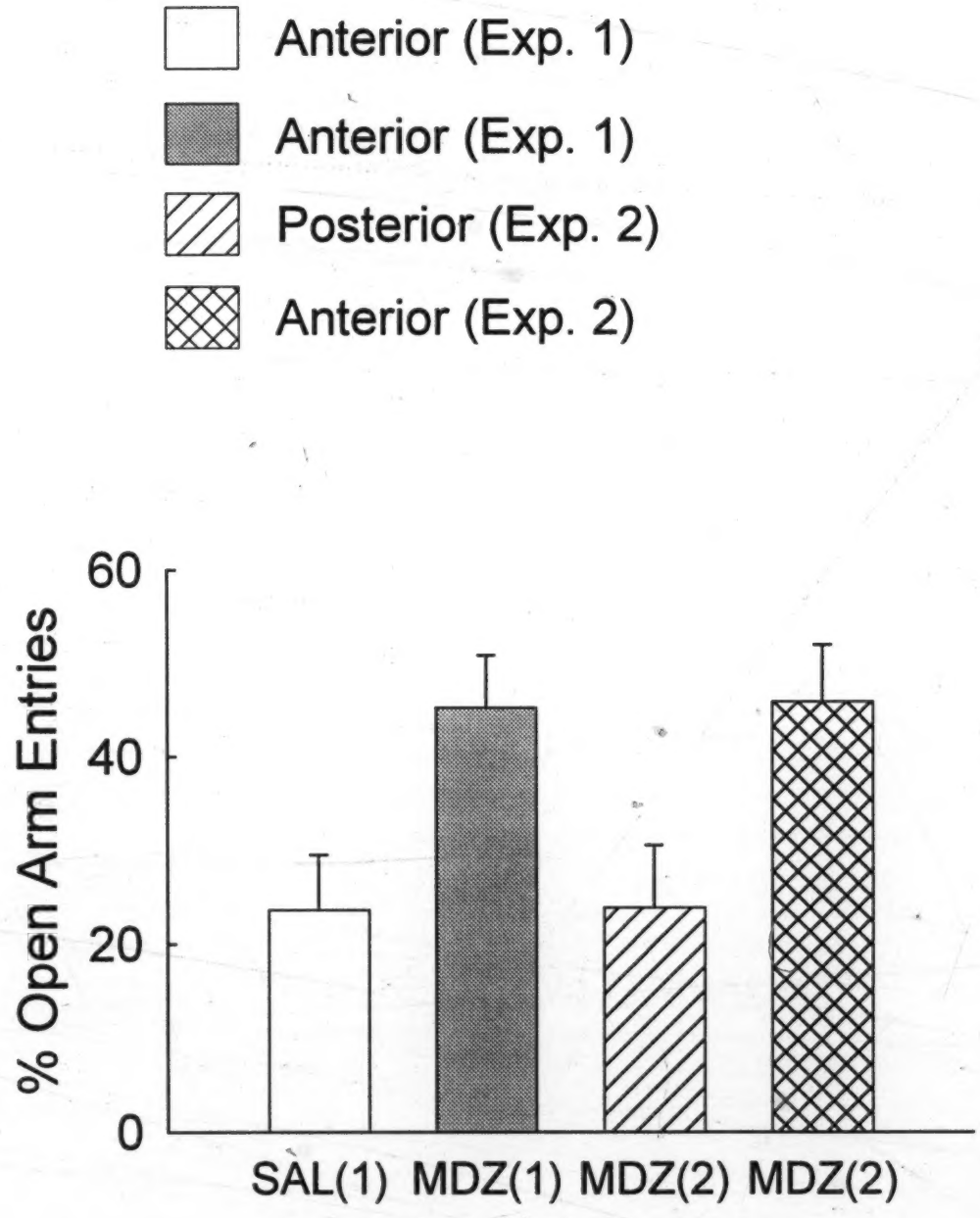
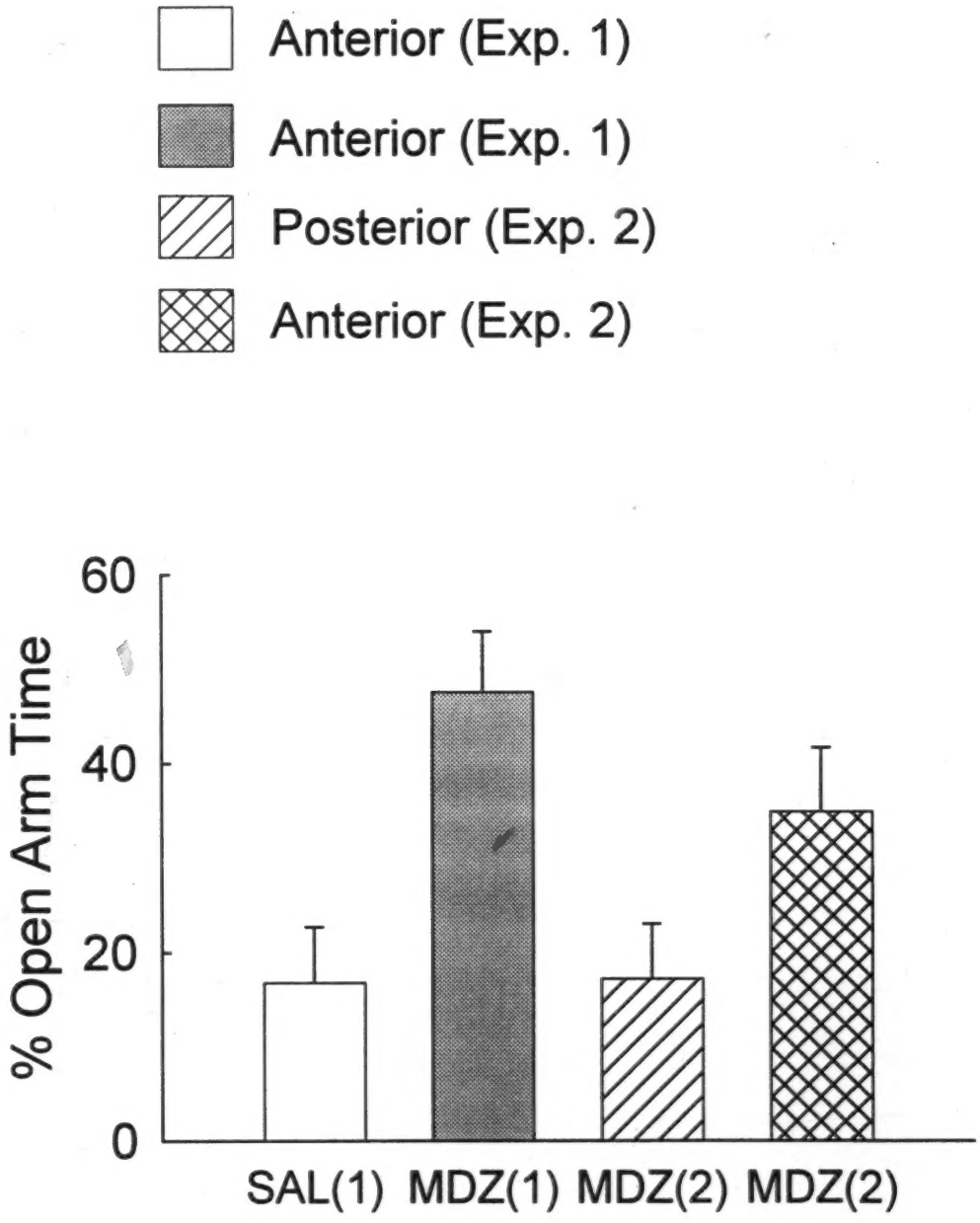

Anterior (Exp. 1)
Anterior (Exp. 1)
Posterior (Exp. 2)
Anterior (Exp. 2)
% Open Arm Entries
60
40
20
0
SAL(1)
MDZ(1)
MDZ(2)
MDZ(2)

Figure 4-8. Mean (± s.e.m.) percentage of time spent in the open arms of the plus-maze after infusions of saline (open bars) or midazolam (stippled bars) into the anterior aspects of the dorsal hippocampus (Experiment 1), and infusions of midazolam into the posterior (diagonal bar) or anterior (hatched bar) aspects of the dorsal hippocampus (Experiment 2).


Anterior (Exp. 1)
Anterior (Exp. 1)
Posterior (Exp. 2)
Anterior (Exp. 2)
% Open Arm Time
60
40
20
0
SAL(1)
MDZ(1)
MDZ(2)
MDZ(2)

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Chapter 5

General Discussion and Conclusions

General findings and implications

Microinfusions of different pharmacological compounds into the septal nucleus or dorsal hippocampus suppressed different fear behaviors in two animal models of anxiety. Septal infusions of the 'inhibitory', 5-HT_{1A} receptor agonist, R(+)-8-OH-DPAT profoundly suppressed rats' burying behavior in the shock-probe burying test, without altering their normal open-arm avoidance in the elevated plus-maze. These findings were unexpected, because suppressing or eliminating activity in the septum typically reduces fear responding in both tests. Nevertheless, septal regulation of these different fear reactions was similarly dissociated following local application of EAA antagonists into the septal nucleus. Specifically, septal infusions of the NMDA receptor antagonist, AP-5 dramatically reduced burying behavior but did not alter open-arm avoidance, whereas septal infusions of the non-NMDA receptor antagonist, CNQX reduced fear responding in both tests. In addition to these pharmacological dissociations, rats' fear responses in the plus-maze and burying tests were dissociated neuroanatomically; i.e., dorsal hippocampal infusions of either R(+)-8-OH-DPAT or the benzodiazepine receptor agonist, midazolam selectively increased open-arm exploration in the plus-maze, without altering fear responses in the burying test. In a subsequent experiment, septal infusions of L-glutamate selectively reduced the open-arm exploration of rats previously infused with midazolam in the dorsal hippocampus, suggesting that the anxiolytic effects of inhibiting dorsal hippocampal activity may be due to suppressed release of glutamate in the septal nucleus. Thus, overall it appears that 5-HT_{1A}, NMDA and non-NMDA receptors in the septal nucleus might have specific roles in the regulation of different fear responses. Similarly, it appears that

specific projections coming into the septum from the dorsal hippocampus differentially regulate rats' fear responses in the plus-maze and burying tests.

Methodological limitations

As mentioned in the previous chapters, further work is needed to fully characterize in detail the receptor specificity of the present results. This is especially the case for results obtained using R(+)-8-OH-DPAT, which, although relatively selective for the 5-HT_{1A} receptor, is also known to bind to the 5-HT_7 receptor (e.g., Zifa & Fillion, 1992). Thus, additional work should focus on whether the behavioral effects of dorsal hippocampal or septal infusions of R(+)-8-OH-DPAT are blocked by coadministration of a selective, 5-HT_{1A} receptor antagonist (e.g., WAY100635; Fletcher, Cliffe, & Dourish, 1993). Although I am unaware of any substantive, non-specific binding of either CNQX or AP-5 to receptor sites other than EAA receptors (e.g., Hansen & Krogsgaard-Larsen, 1990) or midazolam, to sites others than BZ receptors (e.g., Mohler & Okada, 1977), firm conclusions regarding the receptor specificity of the current results obtained with these agents requires further investigation; e.g., using coadministration of EAA agonists (such as non-toxic doses of AMPA and NMDA) and a BZ antagonist (such as flumazenil), respectively. Such findings may further rule out non-specific effects associated with drug related deviations from physiological pH and/or osmotic lesions. The absence of such data notwithstanding, histological analysis in the current studies revealed no apparent evidence of tissue damage which might be expected with chemical and/or osmotic lesions. In addition, the behavioral dissociations observed in the current studies further rule out the

possibility of non-specific lesion effects in the septum (i.e., septal lesions reduce fear responses in both the plus-maze and the burying tests).

As discussed in Chapter 2, drug diffusion to a distal structure (e.g., dorsal raphe) would not account for the differential effects of R(+)-8-OH-DPAT in the septal nucleus and dorsal hippocampus. Similarly, drug diffusion would seem a poor account for the behavioral dissociations observed following septal infusions of CNQX or AP-5 and dorsal hippocampal infusions of midazolam. In addition, the open-arm activity of midazolam-treated rats with cannula(e) placements close to but outside the dorsal hippocampus ($n = 5$) did not differ from controls (both p's > 0.50; data not shown), further suggesting that the anxiolytic effects of midazolam, detailed in Chapter 4, were, in fact, mediated in dorsal hippocampus.

Future directions

Despite the complexities of the present results, Gray's (1982) original "septo-hippocampal" theory of anxiety has gained strong empirical support from a pharmacologically validated animal model of anxiety; i.e., the dorsal hippocampus and septal nucleus seem to exert common control over rat's anxiety-related behaviors in the elevated plus-maze. At the same time, the behavioral dissociations obtained in these studies suggest that the role of the septum and hippocampus is specific to particular fear reactions, rather than being a "centre" for the global control of fear or anxiety, *per se*. Although complex, an explanatory model based on a system of distributed, parallel pathways, each specialized for processing a particular aspect of fear or anxiety may

ultimately prove to be more plausible. Indeed, it seems evident that such a system would confer greater adaptive flexibility for effective, defensive responding under different, environmental contexts.

In this regard, extensive ethoexperimental analysis of defensive responding in rats (e.g., Blanchard, Blanchard, & Rodgers, 1990) indicates that a certain set of defensive responses, such as flight, freezing and defensive threat/attack, is elicited by present, discrete threat stimuli (e.g., an approaching predator), whereas a different set of defensive responses, so-called "risk assessment" behaviors, such as visual scanning and elongated body postures punctuated by abrupt withdrawals (i.e., "stretched attend behavior"), is elicited under conditions of "potential" threat (e.g., where a predator *might* be encountered). Although it is not clear whether (or to what degree) open-arm avoidance and burying behavior map onto the Blanchards' schema of responding to "potential" and "present" threats, respectively, these concepts may provide a useful starting point for further research. In fact, a recent reformulation of Gray's original theory posits that the septum and hippocampus act in concert to regulate defensive responses associated with "potential" rather than "present" dangers, per se (e.g., McNaughton, 1995, 1997). According to this revised theory, we might predict that the septo-hippocampus would be involved in the regulation of open-arm avoidance (which might represent an innate, defensive response towards potential predators; see Chapter 1) but not in the regulation of burying behavior (which is clearly directed towards a present, discrete threat stimulus, i.e., the shock-probe). Although such a pattern was observed following local inhibition of the dorsal hippocampus (Chapters 2 & 4), a thorough test of this hypothesis clearly demands

investigation into the role of the ventral hippocampus in the burying response (for further details see Chapter 4).

In addition, to the extent that the elevated plus-maze and shock-probe burying tests measure defensive responses linked to "potential" or "present" threats, respectively, their combined use may be profitable for determining whether (as suggested by the present results) the processing of these particular aspects of fear or anxiety is governed by functionally distinct, parallel pathways. For example, as indicated in Chapter 4, future studies should be aimed at delineating the role of distinct subdivisions within in the lateral septum (i.e., LSr and LSc) and their respective target sites in hypothalamus (e.g., perifornical region and supramamillary bodies) in rats' fear responses in these tests.

This said, it is important to note that the apparent distinction between "potential" and "present" threats, at least in the case of the burying test, may be in need of further refinement. This is because, despite the "presence" of a discrete threat source, risk assessment behaviors normally elicited by "potential" threats (see above) *are* nonetheless evident in the burying test. Specifically, burying behavior is typically preceded by a variable and often lengthy interval (5 - 10 min), characterized by risk assessment behaviors, such as stretched attend postures directed towards the shock-probe (for further details see Treit, Menard, & Pesold, 1994). Although the exact relationship between risk assessment and burying behaviors requires further study, these responses may be respectively linked to "potential" and "actual" threats that are physically present in the animal's environment. In other words, rats might assess the degree of threat posed by the probe prior to engaging in burying behavior. Furthermore, there is some initial indication

that stimulus conditions favorable to burying might be less favorable to risk-assessment; e.g., casual observations indicate that addition of a "hissing" auditory stimulus in conjunction with probe-contact induced shock may promote immediate, vigorous burying that is not preceded by risk-assessment behaviors. Characterization of the relationship between burying and risk-assessment behaviors in the burying test may reveal situational variables which selectively constrain the expression of each of these different responses. If so, then subtle variations in the shock-probe burying test may permit a more refined investigation into how the brain evaluates threatening stimuli and integrates this information into adaptive, situationally specific, defensive responding. This knowledge may, in turn, yield insight into both normal and pathological fears or anxieties.

Table 5-1. Summary Table

Chapter-Experiment	Drug	Effective dose (μg)	Site	Plus-Maze effect	Shock-Probe effect
2-1	R(+)-8-OH-DPAT	5, 10	Septum	nil	anxiolytic
2-2	R(+)-8-OH-DPAT	10	Septum	nil	anxiolytic
2-2	R(+)-8-OH-DPAT	0.1, 10	d. Hipp.*	anxiolytic	nil
3-1	CNQX	5	Septum	anxiolytic	anxiolytic
3-1	AP-5	5	Septum	nil	anxiolytic
3-2	AP-5	5	Septum	not tested	anxiolytic
4-1	Midazolam	10	d. Hipp.	anxiolytic	nil
4-2	Glutamate	3.5	Septum	nil	not tested
4-2	Midazolam/ Glutamate	10/ 3.5	d. Hipp./ Septum	reversal**	not tested

*Dorsal Hippocampus
**see text

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